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SOME INTERACTIONS OF NITROGEN MUSTARDS WITH CONSTITUENTS OF HUMAN BLOOD SERUM

J. H. LINFORD

Department of Biochemistry, University of Manitoba

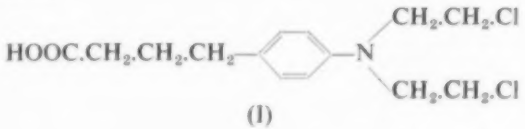
(*Received* 8 *February* 1961; *accepted* 1 *July* 1961)

Abstract—Three compounds of the nitrogen mustard class: *p*-N : N-di (B-chloroethyl) aminophenylbutyric acid, (I), 4-N : N-di (B-chloroethyl) amino-2-methyl-2′-carboxyl azobenzene, (II), and N : N-di (B-chloroethyl)-N′ : O–propylenephosphoric acid ester diamide, (III), have been studied. The rates of their hydrolyses in aqueous solutions and the rates of chemical condensation with proteins of human blood serum *in vitro* have been measured. These reactions proceed more rapidly with compound (I) than with (II) and (III). The compounds (I) and (II) are firmly adsorbed onto protein. They do not react with the bilirubin or lipochrome constituents of serum. The rate of hydrolysis of Chlorambucil is decreased in the presence of serum.

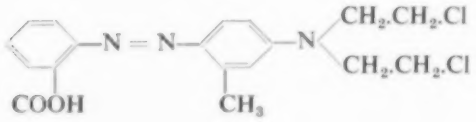
INTRODUCTION

THE chemical properties of the nitrogen mustards have been studied by a number of authors. Ross[1] compared the rates of hydrolysis of a number of nitrogen mustards in acetone–water solvent; the rate of hydrolysis increased in parallel with the ability of these compounds to cause regression of the Walker rat tumour. Stacey *et al.*[2] and Alexander and Cousens[3] reacted bovine serum albumin (BSA) in 0·5 M sodium bicarbonate solution with concentrations of alkylating agents in excess of the number of reactive carboxyl groups present in the protein. A 3% solution of crystallized BSA was made 0·12 M with respect to the bifunctional alkylating agents that were used. The rates of condensation were not determined. About 50 per cent of the available carboxyl groups were esterified by the nitrogen mustards. These authors demonstrated that all the different types of alkylating agents tested esterify carboxyl groups, only the epoxides react extensively with amino groups.

The present paper reports on investigations of some reactions of the following three drugs, (I), (II) and (III) of the nitrogen mustard type with dilute alkalis, serum proteins and bilirubin.

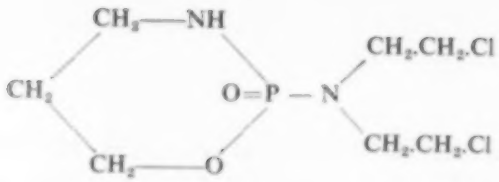


(I)



(II)

A

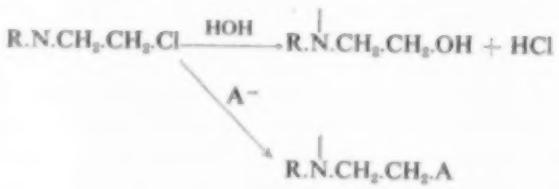


(III)

The molecular weights are as follows: acid form of (I), 304; sodium salt of (II), 402; (III), 261.

These compounds are in clinical use under the names of: (I) CB 1348 and Chlorambucil; (II) CB 1414; (III) B 518 and Endoxan. Compounds (II) and (III) differ from (I) in that they are considered to require modification of the molecule by body processes before the terminal chlorine atoms become reactive.[4–7]

The carboxylic compounds CB 1348 (I) and CB 1414 (II) are moderately soluble in water in the form of the sodium salt of the acid. B 518 is readily soluble (III). These compounds react with anions in solution to form esters; when the anion is an hydroxyl ion the alcohol derivative of the mustard is formed. The former process is usually referred to as alkylation of the acceptor molecule, the latter process as hydrolysis of the alkylating agent. The reactions may be represented by the general equation:



(IV)

The phenomena that were investigated included the absorption spectra in suitable solvents and the degree to which hydrolysis occurred under various conditions. These properties were useful for detection and for measurement of amount of the compounds. Studies of hydrolysis rates were made as quantitative knowledge might be of value in interpreting clinical results. It was also necessary to know how to prepare and to preserve solutions of the drugs in the active and hydrolysed forms.

The extent to which the alkylating agents reacted with the constituents of blood serum, for example by esterification of the carboxyl groups of protein, was of interest because this may be a source of loss of the active drug administered to patients. Furthermore, quantitative studies of these reactions would provide information on the activities of these drugs with respect to compounds of biological importance. Studies on the degree of adsorption of this type of drug to the colloidal protein phase of serum were undertaken because such an occurrence might limit the distribution of the drug.

REAGENTS AND APPARATUS

Absorption spectra were measured with a Hilger Uvispek photoelectric spectrophotometer. Titration procedures and pH measurements were made with a Beckmann H2 glass electrode pH meter. Ultrafiltration was carried out under nitrogen pressures of 40 lb/in^2 through "Visking" cellulose acetate membrane.

Volumetric solutions of 0·025 N sodium hydroxide and 0·050 N hydrochloric acid were prepared. For this purpose British Drug Houses Volumetric Solution 0·1 N hydrochloric acid was used as the primary standard. Concentrations of 0·5 M and less of sodium bicarbonate were prepared. Absolute ethyl alcohol was used as a protein precipitant. Solutions of 0·3 N barium hydroxide and 5 per cent zinc sulphate heptahydrate were prepared for use as a neutral protein precipitant.[8] These solutions were standardized relative to one another with the pH-meter by titrating the barium hydroxide solution into the zinc sulfate solution. One solution was adjusted by dilution, if necessary, so that equal volumes of each yielded a mixture having a pH value between 7 and 8. It was important to titrate the barium hydroxide into the zinc sulfate, as the former when present in excess appeared to be adsorbed onto the barium sulfate–zinc hydroxide precipitates. Bilirubin was obtained from Homburg of Frankfurt. Compound (I) was obtained from the Chester Beatty Institute, London, and from Burroughs Wellcome & Company; Compound (II) was obtained from the Chester Beatty Institute; Compound (III) was obtained from Asta-Werke A.G. The human blood serum was obtained by pooling the excess from samples taken for routine laboratory examinations. It was available from 2 to 3 hr after withdrawal from the patients.

EXPERIMENTAL TECHNIQUES

Detection and measurement of alkylating agents

In aqueous solution as the sodium salt, compounds (I) and (II) possess characteristic absorption spectra which served for their identification and measurement. Compound (I) has a maximum at 258 mμ of optical density 1·55 for a concentration of 30 μg per ml. Compound (II) has a maximum at 410 mμ of optical density 1·75 for a concentration of 30 μg per ml. These values are not affected by changes in the pH of the solvent from 7 to 11·5, nor are they affected by changes in sodium bicarbonate content from 0·025 M to 0·5 M.

In alcohol solution the optical density of (I) is increased to 1·85 but the curve shape is changed very little. The absorption maximum of (II) is shifted to 395 mμ with optical density 2·00. These values refer to concentrations of 30 mμ per ml.

The optical densities, to the measurable limit, were proportional to the concentrations of (I) and (II). Measurements of solutions that had been filtered or extracted from blood serum were compared to blank procedures of the same type carried out in the absence of the drug. Control solutions of the drug in aqueous solvent were processed in the same way. The absorption difference between a drug solution and its blank was compared with the controls by the method of Allan.[9]

Titration methods were also applicable because compounds (I), (II) and (III) are capable of undergoing hydrolysis in alkaline solution; equation (IV) refers. A sample of drug was allowed to hydrolyse completely in the presence of 10 ml of 0·025 N sodium hydroxide. The difference between the amount of standard (0·050 N hydrochloric acid required to titrate the alkali alone and that required to titrate

the alkali plus the added sample was equivalent to the amount of acid liberated. The conditions that brought about complete hydrolysis of each compound are discussed below.

Rates of hydrolysis in aqueous solutions

The rates of hydrolysis were ascertained by measurements of the acid released, equation (IV). Solutions of 75 mg (I), or of 100 mg (II), or of 65 mg (III) in 50 ml of alkaline solvent were prepared and placed in a constant temperature bath. Immediately, and at measured intervals of time, 10 ml volumes were withdrawn, pipetted onto crushed ice in a beaker and titrated at once with 0·050 N hydrochloric acid. The pure solvent was also titrated. The decrease with time in the amount of standard acid required to titrate each 10 ml sample to pH 6·5 ,was equivalent to the amount of acid liberated in the solution in that time. The solvents are listed in Table 1. In some instances the drug was dissolved in alkali and the solution was quickly adjusted to a lower pH for the rate experiment.

The above solutions contained $0{\cdot}50 \times 10^{-4}$ mole of drug in 10 ml. The acid released during hydrolysis did not alter appreciably the pH of 0·025 N sodium hydroxide or bicarbonate in the course of the experiments. Mixtures of sodium hydroxide and hydrochloric acid that were used to obtain pH values of 7·5 and 5 were appreciably reduced in pH by the amount of acid released from the drug. The drugs were allowed to react with these solvents in separate 10 ml volumes, the pH of which was adjusted to the starting value whenever it dropped 0·5 units.

Rates of alkylation in blood serum

The amount of compound (I), (II) or (III) which had condensed with serum protein, at a given time, was determined by alcoholic precipitation of the protein. Of the initial amount of drug, the fraction that was chemically combined was precipitated with the protein but the free drug remained in the supernatant alcohol. The amount in alcohol solution was determined by one of two methods.

For spectroscopic measurement of rates of alkylation, a 10 mg quantity of (I) or (II) was dissolved in 0·5 ml of 0·5 M sodium bicarbonate at 37 °C and diluted at once to 10 ml with serum at 37 °C. Immediately and at measured intervals of time 0·5 ml of the serum solution was diluted to 5·0 ml with ethanol. The mixture was stirred and centrifuged, then 1·0 ml of the supernatant was again diluted to 5·0 ml, or as necessary, with ethanol. The concentration of drug in the final solution was determined spectroscopically.

To study the effect of increased bicarbonate concentration, the drug was dissolved directly by shaking in 10 ml of serum made 0·5 M with respect to sodium bicarbonate. Measurements were made also on solutions of 150 mg of drug in 10 ml of serum, by an additional ten-fold dilution of the supernatant with ethanol.

Experiments were also carried out with the hydrolysed form of (I). Eight milligrams were dissolved in 2·0 ml of 0·5 M sodium bicarbonate and allowed to stand at 37 °C for 2 hr, by which time the chlorine atoms were replaced by hydroxyl groups. Eight millilitres of serum were then added. Over a period of 24 hr at 37 °C, samples were tested by alcohol extraction for content of free drug as described above.

In all methods, standard solutions were prepared with water in place of serum and controls were prepared with serum but no alkylating agent.

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The second method of determining the quantity of drug in the alcohol supernatant, based on titration measurements, was developed for two reasons. It was used to study reactions of compound (III) which in solution was transparent to ultraviolet and visible light. Again, whereas light absorption measurements, when applicable, yielded values of both hydrolyzed and unreacted active alkylating agent that remained in solution, titration measurements would determine only the amount of unreacted active alkylating agent. A comparison of results obtained by light absorption methods and by titration methods would indicate the rate of hydrolysis of the drug in the presence of serum protein and the buffering salts.

To measure with the titration method the rate of alkylation of serum protein by (III), 65 mg were dissolved in 1·0 ml water and made up to 10 ml with serum maintained at 37 °C; the serum was $2{\cdot}5 \times 10^{-2}$ M with respect to the drug. Immediately and at measured intervals of time 2·0 ml of the serum was pipetted into 8·0 ml of ethanol. The mixture was stirred for 2 min and centrifuged. The precipitate was washed with 2·0 ml ethanol. The combined supernatants were poured into a conical flask and 10 ml of 0·025 N sodium hydroxide was added. The mixture was heated in a bath of boiling water for 60 min. A distillation head and condenser were fitted to collect the alcohol. Titration of the solution in the flask was then carried out with standard (0·052 N) hydrochloric acid. Control titrations were made on serum samples in the absence of the drug. The difference between the amount of acid required to titrate the zero-time sample and that required to titrate a control of serum only, was equal to 100 per cent of the drug in solution in the serum. Using the quantities and concentrations of the experiments this was 2·0 ml of 0·052 N acid. As alkylation proceeded the difference decreased if less drug was present in free solution in the active form.

The rate of alkylation of serum protein by compound (I) was measured by the titration method and by absorption spectroscopy. Compound (I) (215 mg) was dissolved in 3·0 ml of 0·5 M sodium bicarbonate and made up to 25 ml with serum at 40 °C. The serum was, therefore, $2{\cdot}85 \times 10^{-2}$ M with respect to (I) and 0·085 M with respect to sodium bicarbonate, assumng an initial bicarbonate content of 0·025 M. Two-milliliter samples were withdrawn and extracted for titration measurements as described for compound (III) except that hydrolysis in the alcohol–sodium hydroxide solution was carried out at 65 °C for 2 hr. In the presence of bicarbonate, the point of neutralization of the sodium hydroxide was taken as pH 8·5. For each 2·0 ml sample of serum withdrawn for titration, an additional 0·4 ml was also withdrawn and pipetted into 3·6 ml of ethanol. This was again diluted fifty-fold and the amount of (I) in solution was measured spectroscopically. Control titration and absorption measurements were carried out on a 25 ml volume of the serum maintained at 40 °C with no drug.

The drug was also measured spectroscopically in the chemically bound form. The protein precipitate from each 0·4 ml sample of serum was dissolved in 2·5 ml of 0·025 N sodium hydroxide and further diluted twenty-five-fold with water. The minimal quantity that could be detected was limited by the absorption of the protein. The maximum optical density due to protein that could be permitted was approximately 1·2, which is the value obtained when protein from 0·4 ml serum was dissolved in 15 ml of alkaline solvent. To detect (I) it must be present in concentrations of 5–10 μg per ml; thus about 100 μg must be combined in 0·4 ml of serum.

Physical binding of CB 1348 (*I*) *and CB* 1414 (*II*) *to protein*

To investigate the occurrence of physical binding when compounds (I), (II) or (III) were in solution in serum, a small fraction of the solvent phase was separated from the colloidal protein phase by ultrafiltration through "Visking" cellulose acetate membrane. The amount of drug present in the filtrates from (I) and (II) was determined spectroscopically; that from (III) was measured by titration.

A series of concentrations of each drug was prepared in 10 ml volumes of serum. Two millilitres of filtrate were collected in a graduated tube in 6 hr. Experiments with (II) were carried out at 40 °C and at 5 °C, with (I) at 5 °C. No alkylation occurred during this time. The initial concentrations of drugs (I) and (II) in serum were checked by an alcohol extraction as described above. A second 0·5 ml of the serum solution was left in proximity to the filter during the experiment and the free drug concentration was checked again at the end of the filtration period.

In the case of compound (I), experiments were also made with the hydroxyl form of the drug. Five milligrams were dissolved in 2·0 ml of solvent which was 0·025 N sodium hydroxide and 0·13 M sodium chloride. 1·0 ml of the solution was immediately diluted to 10 ml with serum at 5 °C, the remaining 1·0 ml was kept at 37 °C for 2 hr to hydrolyze, then made up to 10 ml with serum. The initial concentrations were checked by alcohol extraction and both solutions were ultrafiltered.

To measure the degree of physical binding of (III) to serum protein, three solutions were prepared of 40 mg, 20 mg, and no drug in 2·0 ml of water, which were made up to 12·0 ml with serum. From each solution 2·0 ml was removed for assay, the remaining 10 ml were subjected to ultrafiltration to obtain 2·0 ml of filtrate from each sample. The six 2·0 ml samples were mixed with 8·0 ml of ethanol and assayed for drug content by hydrolysis and titration as described above.

The degree of binding to protein as determined by the ultrafiltration method was the average result of 6 hr of contact. To determine whether rapid binding did occur, the protein in the serum was precipitated chemically immediately after the addition of drugs (I) or (II). The Somogyi method was used to keep the pH above 7·5. Volumes of serum–drug solution of 2·5 ml were stirred into 5·0 ml of 0·3 N barium hydroxide solution. Five millitres of an equivalent concentration of zinc sulphate were added and the mixture was stirred for 2 min, while 2·5 ml of serum and 2·5 ml of an aqueous standard solution of drug were treated in the same manner. A second 2·5 ml of the standard was diluted with 10 ml of water. After centrifuging, the concentrations of drug in the supernatant liquids were determined spectroscopically.

Interaction of CB 1348 (*I*) *with bilirubin and lipochrome constituents of serum*

The distribution of the bilirubin and lipochrome constituents, which are responsible for the yellow color of serum, was of interest in connection with the methods of precipitating protein from serum. When a fresh solution of (I) in serum was treated with the Somogyi reagent the drug was removed and so were the yellow constituents, along with the protein. When a similar fresh solution was heated with alcohol to remove protein, the drug remained in the supernatant as did also the yellow constituents. Experiments were carried out to determine whether a direct reaction was taking place between (I) and the lipochrome materials.

For the first experiment, 10 ml of serum was pipetted into 40 ml of ethanol, the mixture was stirred and centrifuged, the supernatant was decanted into a flask and

distilled under reduced pressure until about 6 ml of liquid remained. This was made up to 9 ml with water. To this yellow solution was added 1·0 ml of a solution which contained 1·5 mg (I) in 0·5 M sodium bicarbonate; 2·5 ml of this mixture was treated with 5·0 ml of barium hydroxide and 5·0 ml of zinc sulphate solution as for the removal of protein. The inorganic precipitant removed the bilirubin and lipochrome fractions completely, leaving a clear, colorless supernatant. In the same way, 2·5 ml of a standard aqueous solution of (I), which contained 30 μg per ml, was treated. The concentrations of drug in the supernatant liquids were measured spectroscopically.

For the second experiment a solution of 80 mg per cent bilirubin in 1·85 per cent sodium carbonate was prepared, which also contained 1·5 mg (I) in 10 ml. This solution and an aqueous standard of (I) were cleared by the Somogyi reagent as above and the colorless supernatants were examined spectroscopically.

RESULTS AND DISCUSSIONS

Rates of hydrolysis in aqueous solutions

Solution in alkali of the acid form of (I) resulted in the immediate release of 1 mole of hydrogen ion per mole of drug as the sodium salt was formed. The slower hydrolytic process was made evident by production of two additional moles of hydrogen ion. The titration measurements which indicated these processes in 0·025 N sodium hydroxide at 37 °C are shown in Fig. 1. During each titration the sample became cloudy

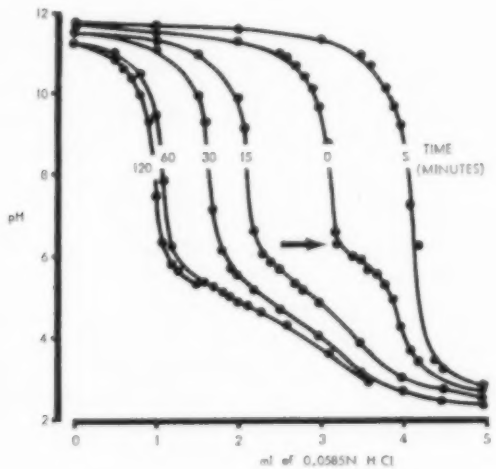

FIG. 1. Measurement of hydrolysis at pH 11·5 of CB 1348 in 0·025 N sodium hydroxide by titration of 10 ml volumes of 0·50 × 10^{-3} M solution with standard acid. (S) is solvent only. Numbers on curves are time in minutes at 37 °C.

at pH 6·2 because of formation of the non-ionized acid (I). This occurrence was shown also by the break in the titration curve at pH 6·2 as indicated by the arrow, Fig. 1. Similar experiments with compounds (I), (II) and (III) in a number of solvents yielded reactions which were all first order. Compound (II) began to precipitate from

solution as the acid form at pH 7·5. The degree of dependence of the rate constant on the temperature and the ion species present is shown in Table 1.

The rate constants of CB 1414 (II) and B 518 (III) at 37 °C are one-fiftieth or less that of the CB 1348 (I) reaction. The temperature coefficient of reaction of (1) is large; no appreciable hydrolysis occurred when stored at 5 °C for 24 hr. From the rate constants at 25 °C and 37 °C the activation energy of the hydrolytic reaction of (I) was calculated to be 23 kcal/mole.

The presence of 0·13 M sodium chloride did not alter significantly the hydrolytic reaction rate of (I). This indicates the reaction to be irreversible and it must be accompanied by a large decrease in standard free energy.

TABLE 1. EFFECT OF TEMPERATURE, pH AND COMPOSITION OF SOLVENT ON THE RATE OF HYDROLYSIS OF CERTAIN NITROGEN MUSTARDS

Drug	Temperature (°C)	pH	Solvent	Rate constant k
CB 1348	37	5	0·025 N NaOH 0·050 N HCl	0
CB 1348	37	7·5	0·025 N NaOH 0·050 N HCl	0·008
CB 1348	37	11·5	0·025 N NaOH	0·03
CB 1348	37	11·5	0·025 N NaOH 0·13 M NaCl	0·027
CB 1348	25	11·5	0·025 N NaOH	0·006
CB 1348	37	8·5	0·025 M $NaHCO_3$	0·04*
CB 1414	37	11·5	0·025 N NaOH	0·0005
B 518	37	11·5	0·025 N NaOH	0·0004

* Alkylation of bicarbonate anion to form an insoluble condensation product also occured.

At 37 °C, hydrolysis of (I) in 0·025 N sodium hydroxide, and in 0·025 M sodium bicarbonate, was complete in 120 min. In 0·025 N sodium hydroxide the hydrolysis of (1) at 60 °C was complete in 30 min; that of (III) at 100 °C was complete in 60 min. Rate constants were not measured at these latter two temperatures but this information was used in the hydrolytic and titration methods of assay.

Rates of alkylation in blood serum

The rate of condensation of (I) with serum at 37 °C is shown in Fig. 2(a). These data represent the differences in the amounts recovered in the alcohol soluble form, as measured spectroscopically. The zero time recoveries corresponded closely to 100 per cent concentration; in comparison with a standard aqueous solution they showed a small increase which corresponded to a 5 per cent decrease in solvent volume caused by precipitation of the protein. The reaction rate was not affected by the change from 0·025 M to 0·5 M concentration of sodium bicarbonate in the serum.

The logarithm of the concentration of unreacted compound (I) is plotted against

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time in Fig. 2(b). The graph is a curve which may consist of two straight lines. Assuming the first linear portion to represent the reaction of a small amount of drug with excess of protein, the rate constant k is 0·0010 at 37 °C. At 25 °C the rate constant dropped to 0·0003. At 5 °C no detectable degree of alkylation occurred in 24 hr. The reaction has a marked temperature coefficient, the activation energy is 25 kcal/mole.

Of the compound (I) that was hydrolyzed prior to mixing with serum, 96 per cent was recovered after 17 hr at 37 °C. This confirmed that the decrease in the amount of (I) extractable by alcohol was due to an alkylation reaction.

Solutions in serum of CB 1414 (II) of concentration $4{\cdot}0 \times 10^{-3}$ M were subjected to alcohol extraction as for (I). By spectroscopic measurements, no appreciable change in the concentration occurred over a period of 24 hr at 37 °C. No alkylation of serum protein had occurred.

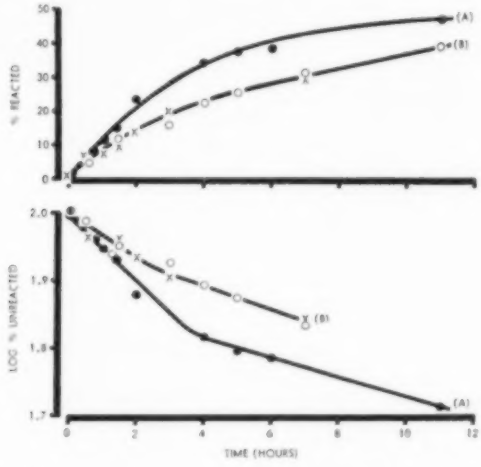

FIG. 2(a). Graphs showing the rate of the alkylation reaction of CB 1348 with serum protein, at 37 °C
FIG. 2(b). Data of Fig. 2(a) plotted as logarithm of percentage of unreacted CB 1348 in relation to time in hours. Curves A, concentration $5{\cdot}0 \times 10^{-2}$ M; curves B, concentration $0{\cdot}26 \times 10^{-2}$ M CB 1348 in serum. ○-○-○ and -●-●-● serum 0·5 M with respect to added bicarbonate. ×-×-× serum 0·025 M with respect to added bicarbonate.

Solutions in serum of B 518 (III) at concentrations of $2{\cdot}5 \times 10^{-2}$ M were subjected to alcohol extraction, By the titration method, recoveries over a period of 24 hr at 37 °C were 98–102 per cent. No alkylation of serum protein had occurred and no hydrolysis of the drug had taken place.

The rate of alkylation of serum protein by compound (I), the only one of the three drugs to react at a measurable speed, was determined by both titration and absorption measurements on the alcohol extracts. The difference between the two rate curves as plotted in Fig. 3 is the rate of hydrolysis of (I) ($2{\cdot}85 \times 10^{-2}$ M) at 40 °C in blood serum made 0·085 M with respect to sodium bicarbonate. In the presence of the serum 10 per cent of the drug was hydrolysed in 6 hr; in aqueous 0·025 M bicarbonate hydrolysis was complete in 2 hr. We may assume either that the thermodynamic activity

of the bicarbonate was reduced, or that the drug was protected, by the presence of serum protein.

The rate of alkylation by (I) as shown in Fig. 3, curve A was checked by re-dissolving the protein residues, followed by spectroscopic measurement of the amount of drug chemically combined with the protein. The results corresponded exactly with curve A on a relative scale. On an absolute scale, the amount condensed with the protein residue at 22.5 hr corresponded to 51 per cent of the added drug; curve A records 65 per cent of the added drug in the combined form at this time. The absorption coefficient of the drug in the chemically combined form may be less than the value in the free form; an optical density of 1·03 for a drug concentration of 30 μg per ml, in the combined state, would yield a rate curve identical with curve A.

No alkylation reaction with whole red cells occurred at 37 °C when (I) was incubated for 20 hr in a suspension of red cells in 1 % sodium chloride of volume equal

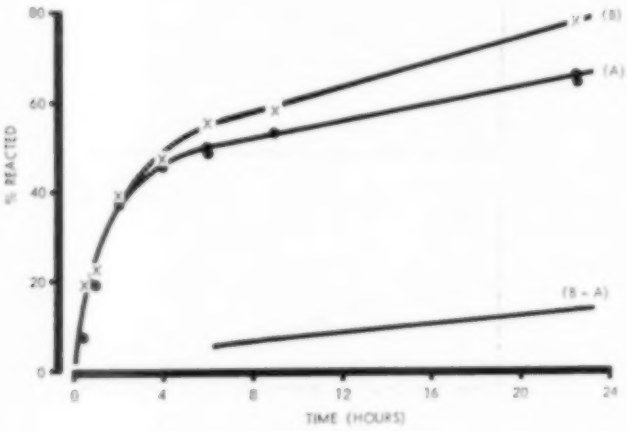

FIG. 3. Comparison of measurements of rate of reaction of CB 1348 $2{\cdot}85 \times 10^{-2}$ M in blood serum (0·085 M sodium bicarbonate) at 40 °C. Curve A, rate of alkylation reaction by absorption method; curve B, rate of alkylation reaction plus hydrolysis reaction by titration method. The numerical difference between curves B and A is plotted as measured from the graphs.

to the serum removed. At the end of the period 100 per cent of the drug present was recovered by alcohol extraction.

Interaction of (I) with bilirubin lipochromes

Solutions of (I) in aqueous bicarbonate, in the presence of a lipochrome extract of serum and in the presence of bilirubin, were treated with the Somogyi reagent. The solutions were rendered colorless by the reagent but the absorption of the supernatant liquid did not differ significantly from aqueous standards used as controls. No interaction of (I) with the bilirubin, or the lipochrome fractions of serum, could be detected.

Physical binding of CB 1348 (I), *CB* 1414 (II) *and B* 518 (III) *to protein*

The results of the ultrafiltration of solutions of compound (II) in blood serum are

shown in Table 2 and Fig. 4. Table 2 refers to measurements made over a wider range of concentrations, Fig. 4 refers to a second series of experiments over a range of lower cencentrations. No alkylation reaction occurred under the experimental conditions.

The values in Table 2 show that 95–99 per cent of the drug was not in free solution. As the total amount present could be extracted by alcohol it may be assumed that the drug was adsorbed to the protein. The degree of adsorption was the same at 5 °C and 40 °C. The relative amount of drug in the bound form decreased rapidly at drug concentrations greater than 15 mM.

TABLE 2. DISTRIBUTION OF SODIUM SALT OF CB 1414 (II) IN BLOOD SERUM BETWEEN PROTEIN-BOUND AND FILTRABLE STATES

40 °C				5 °C			
Bound (m-moles/l.)	(%)	Filtrable (m-moles/l.)	(%)	Bound (m-moles/l.)	(%)	Filtrable (m-moles/l.)	(%)
1·02	99·3	0·007	0·7				
2·38	99·4	0·014	0·6				
3·46	99·2	0·029	0·8	3·24	99·3	0·022	0·7
3·67	98·9	0·040	1·1	5·88	99·5	0·031	0·5
4·70	99·2	0·039	0·8	5·97	98·8	0·069	1·2
5·28	98·7	0·066	1·3	11·7	98·2	0·210	1·8
9·75	98·6	0·135	1·4	13·6	96·1	0·528	3·9
12·8	97·0	0·380	0·3	13·9	96·5	0·488	3·5

The results of the second series of experiments are plotted in Fig. 4(a), curve A, the shape of which corresponds to an adsorption isotherm. This is borne out by the plot in Fig. 4(b), curve A, which shows an approximately linear relationship of the logarithms of the concentrations in the solid and solution phases. At concentrations above 4 mM the relative amount bound to protein appeared to increase. This may indicate the occurrence of denaturation of the protein.

The results of ultra-filtration of solutions of compound (I) in blood serum are shown in Table 3. The experiments were carried out at 5 °C at which temperature no alkylation reaction occurred.

The measurements demonstrated that CB 1348 (I) was adsorbed to a high degree in the presence of protein but the amount bound decreased more rapidly with increase of drug concentration than was the case with compound (II). This suggested that the benzene ring was responsible for the adsorption. Also, replacement of the active chlorine atom of (I) by hydroxyl greatly reduced the degree of physical binding.

The ultrafiltration of solutions of B 518 (III) of molarities $1{\cdot}4\times10^{-2}$ M and $0{\cdot}7\times10^{-2}$ M in blood serum yielded filtrates which did not differ appreciably in concentration from the initial serum solutions. No measurable adsorption had occurred, but a reduction of concentration of less than $1{\cdot}0\times10^{-3}$ M would not be detected.

Chemical precipitation of the serum proteins by the Somogyi reagent in the presence of (I) and (II), removed the drugs to a great extent. At concentration $0{\cdot}5\times10^{-2}$ M in serum, compound (I) was removed completely since the resultant spectrum was

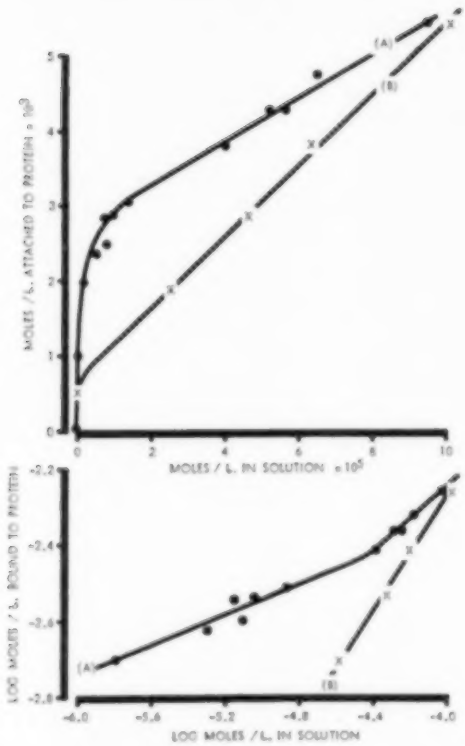

FIG. 4(a). Adsorption isotherms of CB 1414 at room temperature relating molar concentration in free solution to the number of moles bound in one litre of human blood serum.
FIG. 4(b). Date of Fig. 4(a) plotted as logarithm of quantity of CB 1414 bound to protein in relation to logarithm of concentration in free solution. Curves A, equilibrium concentration of free CB 1414 determined by ultrafiltration. Curves B, concentrations of free CB 1414 determined by chemical precipitation of protein.

TABLE 3. DISTRIBUTION OF SODIUM SALT OF CB 1348 (I) IN BLOOD SERUM AT 5 °C BETWEEN PROTEIN-BOUND AND FILTRABLE STATES

Bound (m-moles/l.)	(%)	Filtrable (m-moles/l.)	(%)
0·822	100	0·0	0·0
2·21	97·1	0·066	2·9
3·45	94·9	0·184	5·1
3·88	92·3	0·324	7·7
4·37	92·8	0·338	7·2
6·38	86·8	0·972	13·2
6·90	80·9	1·63	19·1
*0·32	37	0·55	63

* Chlorine atoms in (I) replaced by hydroxyl groups.

indistinguishable from that of serum treated in the absence of the drug (Fig. 5). Only a small change in light absorption occurred when the aqueous control solution was subjected to the Somogyi reagent.

In the control solutions that contained drug but no serum, the salt precipitate decreased the volume of liquid from the 12·5 ml total of drug solution plus reagents to 10·5 ml. When solutions of (I) of concentration greater than 40 μg per ml were treated with the Somogyi reagent a perceptible quantity of drug adhered to the barium sulfate–zinc hydroxide precipitate. Of a total of 375 μg of (I) present in 2·5 ml of solution, 11 per cent was extracted by alcohol from the salt precipitate; on the basis that 89 per cent of the drug remained in the supernatant liquid, the volume of solvent calculated from optical density measurements of (I) agreed with the measured volume of 10·5 ml.

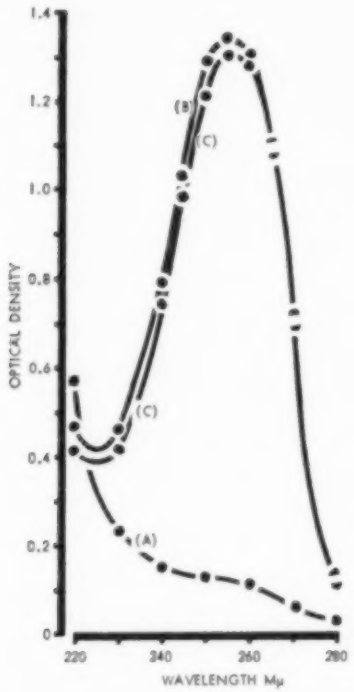

FIG. 5. Graphs showing the adsorption to the protein components in human blood serum of CB 1348 in $0{\cdot}50 \times 10^{-2}$ M solution. Curve A, absorption spectrum of supernatant after precipitation of serum proteins by Somogyi reagents. Curve B, absorption spectrum of supernatant after treatment of aqueous control solution by Somogyi reagents. Curve C, aqueous control solution untreated.

When serum solutions were treated with the Somogyi reagent, the serum–salt precipitate reduced the liquid volume from 12·5 ml to 7·5 ml. The concentration changes were corrected for this decrease of solvent volume.

The protein–salt precipitates were extracted with alcohol. The precipitate from a freshly prepared drug solution yielded 100 per cent of the drug content of the serum.

The precipitate from a solution of (I) in serum which had been incubated at 37 °C for 6 hr yielded 50 per cent of the drug present.

The distribution of (II) between the solvent phase and the colloidal protein phase of serum, as determined by chemical precipitation of the protein, is shown by curves B of Figs. 4(a) and 4(b). Compared to the values obtained by ultrafiltration, a greater proportion was in the solvent phase for the same initial concentration of (II) in the serum. This probably was caused by the dilution of the serum which occurred when the Somogyi reagents were added. In comparison with the values obtained by ultrafiltration, the rate of increase of adsorbed drug with increase of drug concentration was greater. This indicates greater adsorption of (II) to denatured (precipitated) protein than to protein in the native state. The change in the slope of curve A, Fig. 4(b), at concentrations greater than 4×10^{-3} M is in the direction of the slope of curve B. This suggests that high concentrations of (II) act as a protein denaturant.

No adsorption of (I) to red cells occurred. A freshly prepared solution of (I) in whole blood was centrifuged to separate the plasma and red cell components. All the drug was in the serum phase.

CONCLUSIONS

Certain effects of the structures of alkylating compounds, on properties which may be involved in the clinical applicability of these compounds, have been demonstrated. The three compounds of the β-chloroethylamine type that were studied have analogous clinical application but differ among themselves in their reactivities *in vitro* in some respects. Compounds (II) and (III) differ from (I) in that they undergo hydrolysis in aqueous alkaline solution at a much slower rate. In parallel with this difference in reactivity, compound (I) undergoes a condensation reaction with serum protein whereas compounds (II) and (III) do not react appreciably. The same activation energy of 24 kcal applies to both the hydrolytic and alkylation reactions of (I). These reactions appear to be accompanied by a large decrease in standard free energy, because neither the rate nor the extent of the hydrolytic reaction are affected by the presence of an excess of chloride ion, and the alkylation of blood serum by 0·0026 M (I) proceeds in the presence of the chloride content of serum, which is approximately 0·1 M.

As clinical effectiveness must depend upon the occurrence of the alkylation reaction, compounds (II) and (III) may be modified within the body or may react more rapidly with groups other than the carboxyl of protein. In this connection, the hydrolytic and serum protein condensation reactions of compound (I) are probably sources of waste of the administered drug. The half-life of the condensation reaction is about 6 hr; the rate of hydrolysis in the presence of serum protein is relatively unimportant. The condensation reaction has a high temperature coefficient which could conceivably favor specific tissues. *In vitro* no alkylating reaction occurred between compound (I) and the bilirubin or lipochrome components of blood serum, nor with red cells in suspension in saline.

The proteins of blood serum adsorb compounds (I) and (II) rapidly, and at concentrations comparable to those prevailing *in vivo* adsorption is nearly complete. No adsorption of (III) could be detected. The binding forces appear to be van der Walls type since the benzene rings of the drugs are involved and the bonds are broken

by solvents such as ethanol. This physical binding must limit the extent of diffusion of (I) and (II) but at certain locations they must be released to penetrate cell membranes. No adsorption of (I) or (II) to whole red cells could be detected.

The methods which were developed have enabled measurements to be made of the rate and extent of reaction of these alkylating agents with serum protein in colloidal suspension. Applied to other tissues and particles, similar methods may be of use in determining whether such drugs exhibit selectivity of action. The methods are not sufficiently sensitive to investigate reactions of the drugs in concentrations approaching those that may be present during clinical use of the drug (less than 0·03 mg per ml of serum). The titration method of detecting changes in drug concentration required a minimum amount of from 2 to 3 mg (Fig. 1). This amount could be reduced to one-fifth by use of a more sensitive pH-meter fitted with an external short-range scale. Assay of the free drug (I) in serum by alcohol extraction was not applicable to concentrations of less than 0·3 mg per ml of serum; assay of the protein-condensed form in alkaline solution was limited to the same degree. These limitations due to extraneous absorption might be overcome by the use of a double-beam spectrophotometer which would be able to compare the drug solution with a reference solution of low transmission.

Experiments are being made at present to overcome the limitations so that the alkylation reaction of small amounts of (I) with blood serum can be followed to completion and the rate of disappearance of administered drug from the blood may be studied.

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RUBIDIUM AND CREATININE TRANSPORT ACROSS ISOLATED MESENTERY*

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Abstract—The movement of Rb^{86} cations across isolated rabbit mesentery displays the kinetic pattern associated with passive diffusion. The permeability constant can be increased by the application of several drugs. Histamine markedly stimulates the movement of rubidium ions, while epinephrine and acetylcholine are much less effective. By measuring the contemporaneous transfer of the Rb^{86} cation and of un-ionized creatinine, an attempt was made to expose the nature of the permeability barrier. A 10° rise in temperature produced a relatively greater elevation in creatinine permeability, while 5-HT was relatively more effective in enhancing the permeability to rubidium. The differential effects of these two environmental stimuli on the trans-mesenteric movement of two tracer substances indicate that these two stimuli must affect different parts of the transport process.

IN recent years considerable attention has been focused on the transfer of solutes across natural and artificial membranes composed of living cells. The naturally occurring barriers that have attracted the most attention have been the frog skin,[1, 2] toad bladder,[3] gastrointestinal mucosa,[4, 5] and capillary endothelium.[6] Artificial membranes include tumor cells that have been caught by filtration on Millipore filters.[7] In most of these systems the emphasis has been directed toward solutes that are transported across the membranes against electrochemical gradients, particularly sugars,[8] amino acids,[7] and electrolytes.[1–5] There have been no demonstrations, however, of active transport across the capillary wall or across the mesentery. Passive diffusion and facilitated diffusion,[8, 9] while commonly conceded to represent important modes of transport in biological systems, have received little attention in the recent past.

Isolated mesentery is a convenient example of a cellular barrier through which diverse solutes migrate apparently only in the direction of the chemical gradient. Such migration, however, is not a simple phenomenon, as is demonstrated in this report. The original observations[10] were limited to the passage of Rb^{86} cations across the isolated rabbit mesentery. It was shown that the rubidium flux can be modified by a variety of chemical factors, e.g. pH, calcium ion concentration, oxygenation, 2 : 4-dinitrophenol, and mercuric chloride. In many respects the permeability responses of isolated mesentery proved to be similar to the reactions of intact capillaries.[11]

The present report extends this study to include observations on the influence of

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several drugs on the Rb^{86} flux. To learn more about the nature of the permeability process, the simultaneous movements of Rb^{86} and of creatinine have also been determined.

METHODS

In the passage of a substance through a membrane by passive diffusion, the flux dQ/dt is a function of the membrane thickness d, the area of the membrane A, and the concentration gradient across the membrane $(C_1—C_2)$:

$$\frac{dQ}{dt} = \frac{DA(C_1—C_2)}{d} \tag{1}$$

D in this equation[12] is a diffusion constant (cm^2/min).

For any one membrane and any one substance, D and d can be combined to give a permeability coefficient K, which has the units of cm/min. In a situation in which the sink volume and the source concentration of the diffusing substance are held constant (as in our system), the diffusion process can be defined by a definite integral of equation (1):

$$(C_\infty—C_t) = (C_\infty—C_0)e^{-kt} \tag{2}$$

where C_∞ is equal to the sink concentration at equilibrium or to the constant source concentration; C_t is the sink concentration at any time t (min); C_0 is the sink concentration at zero time; and k is the first-order rate constant in reciprocal minutes. The rate constant k was evaluated by the same methods as described previously.[10] The value of the permeability coefficient K is related to this first-order rate constant as follows:

$$K = \frac{kv}{A} \tag{3}$$

where v is the sink volume in cm^3, and A is the area of the membrane in cm^2. In this paper we have presented all of the flux data in terms of the permeability coefficient K. P-values in the tables represent probability levels at which drug-induced changes in K were judged to be significant according to the Student's t.

The general technique employed in measuring the Rb^{86} flux in these experiments was like that used before.[10] The rabbit mesentery free of macroscopic blood vessels and fat was tied onto the end of a glass tube, which was suspended in such a way that the membrane did not touch the walls or bottom of a 10-ml beaker, the contents of which served as the diffusion sink. The sink fluid was pumped continuously in closed system through a small chamber contained in a scintillation well-counter and back to the sink. The pumping not only served to stir the fluid but also permitted continuous monitoring of the changing radioactivity in the sink. Throughout each experiment the volume of the diffusion sink was maintained constant.

Either Krebs–Ringer bicarbonate or a modified Krebs–Ringer phosphate (with half the usual phosphate concentration) was used as the base solution; both were buffered at a pH of 7·4[13]. The apparatus was immersed in a constant temperature bath. The superfusate (source solution) contained tracer amounts of $Rb^{86}Cl$ and in some experiments creatinine (about 8 m-moles/l). Otherwise, the source and sink fluids were identical and had essentially the same osmotic pressure. The apparatus

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was assembled in such manner that no hydrostatic pressure difference existed across the membrane.

When creatinine was measured along with Rb^{86}, the experiment consisted in a series of consecutive periods of about 45 min each. During each period the Rb^{86} was monitored as described above. The creatinine flux was determined by analyzing (alkaline picrate method) the creatinine concentration in the sink fluid at the end of each period. On the assumption that creatinine movement can be described by equation (2) above, K was calculated. The sink fluid was then replaced with an equal volume of base solution containing no creatinine or Rb^{86}, at which time the next experimental period began.

This replacement was accomplished by inserting a large container completely filled with unlabeled base solution into the plastic tube which returned the fluid to the sink. The labeled fluid coming from the well counter was pumped into the bottom of this large reservoir, displacing from the top of it into the sink an equal volume of unlabeled solution. The pumping process was continued for about 5 min until the radioactivity of the sink fell to background. This method of exchanging the sink fluid allowed both the sink volume and the tension on the membrane to remain constant. The next experimental period began at the moment the exchange reservoir was removed from the system.

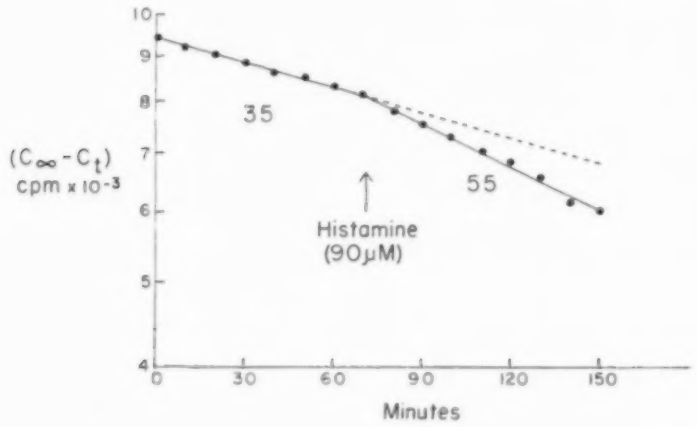

FIG. 1. The effect of histamine on Rb^{86} movement through isolated rabbit mesentery. The difference in radioactivity across the membrane (C_∞—C_t) is depicted on a logarithmic scale. Histamine (90 μmoles/l.) was added at the arrow and the concentration was maintained thereafter. The number under each straight line is the permeability coefficient K calculated for that period.

The drug solutions used in these experiments were always freshly prepared from the dry salts just before use and were introduced into the source reservoir after an appropriate control period or periods. The source solution bathing one surface of the membrane was replaced continuously in order to maintain constant the concentrations of drug and of the penetrating solute or solutes. The drugs used were epinephrine bitartrate, acetylcholine iodide, histamine dihydrochloride, and 5-hydroxytryptamine creatinine sulfate.

RESULTS

Histamine

Because histamine has been shown to be very effective in altering capillary permeability,[11] this drug was tested on the Rb^{86} flux across the isolated rabbit mesentery. As seen in Fig. 1, the result was a prompt and sustained increase in the permeability of the mesentery. The data from this and similar trials (no creatinine present) are summarized in Table 1. A different rabbit was used for each experiment. Although inactive in phosphate-buffered Ringers at 23 °C, histamine at 38 °C appeared to be almost as effective in phosphate as in bicarbonate. In all but one trial (omitted from Table 1), the rise in permeability was significant even at the 0·01 level of significance (Student's *t* test). In general, histamine was as effective in increasing the permeability of mesentery as was 5-hydroxytryptamine (as was reported in an earlier paper).[10]

TABLE 1. DRUG EFFECTS ON THE K FOR Rb^{86} THROUGH ISOLATED RABBIT MESENTERY AT 38 °C

Drug*	Buffer	Control K (cm $min^{-1} \times 10^3$)	Mean %Δ†	Significant at P = 0·05
Histamine	Phosphate	64	+21	2 out of 2 expts.
Histamine	Bicarbonate	41	+41	4 out of 4 expts.
Epinephrine	Phosphate	48	+11	1 out of 1 expts.
Epinephrine	Bicarbonate	45	+21	3 out of 5 expts.
Acetylcholine	Phosphate	63	0	0 out of 2 expts.
Acetylcholine	Bicarbonate	42	+26	2 out of 5 expts.

* The drug concentrations, in μmoles per liter, were as follows: histamine 90, epinephrine 55, and acetylcholine 66.

† In this and other tables, %Δ indicates the percentage change in K that attended exposure to the drug.

Epinephrine

Table 1 also shows that epinephrine increased the permeability of the mesentery in four out of six trials, but the magnitude of the rise was not large. The characteristic red color of adrenochrome (oxidation product of epinephrine) was always seen, sometimes as soon as 30 min after preparing the test solution. There appeared to be an inverse relationship between the magnitude of the permeability response and the formation of the red color as judged visually. Perhaps our difficulty in obtaining reproducible results was due to the destruction of epinephrine, or the ability of the oxidation product to interfere with the action of epinephrine in enhancing the permeability of this tissue, or both.

Acetylcholine

Since acetylcholine is widely believed to promote conduction and transmission in certain specialized tissues by altering their permeability to electrolytes, this drug was tested on the Rb^{86}-mesentery system. There is no evidence in the literature to indicate that acetylcholine alters capillary permeability; the edema formation it produces has

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been ascribed to an increased capillary hydrostatic pressure secondary to arteriolar dilatation.[14, 15]

The data in Table 1 indicate that acetylcholine was not nearly as effective as histamine in stimulating Rb^{86} flux across rabbit mesentery. In fact, statistically significant effects were obtained in only two of the seven experiments. At the present time no physiological or pharmacological significance can be ascribed to these observations with acetylcholine.

5-Hydroxytryptamine (5HT)

The influence of 5HT on Rb^{86} movement alone has been reported before.[10] In the bicarbonate buffer at 38 °C, this drug, in a concentration of 55 μmoles/l. produced consistently a marked increase in the Rb^{86} permeability of the isolated mesentery. In order to investigate the mechanism of this action and to uncover more information about the transport process, we have measured the contemporaneous movement of Rb^{86} and creatinine. Appropriate control experiments demonstrated that creatinine did not modify the Rb^{86} flux.

In Fig. 2 are presented the flux data for a control experiment, together with the values of K calculated for each period. The creatinine data are shown as straight

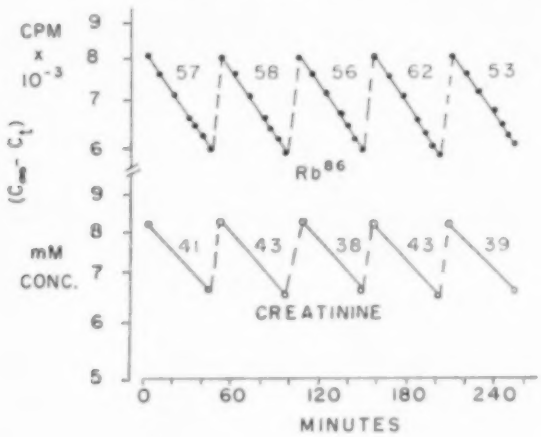

FIG. 2. Control flux data for the simultaneous movement of Rb^{86} and creatinine. See text for a complete explanation.

lines, even though there are only two points from which to construct each line. The assumption underlying this procedure has been discussed above. This analysis makes it possible to calculate values for K, in order that a comparison can be drawn between the permeability to Rb^{86} and to creatinine. In each case, the value for Rb was larger than that for creatinine. In this and other experiments, the mean difference was about 40 per cent of the creatinine value. Fig. 2 demonstrates also the stability of the Rb^{86} flux during each individual period, as well as the reproducibility of both the rubidium and creatinine data collected over several consecutive periods.

When the permeability was altered by the addition of 5HT, the fluxes of Rb^{86} and of creatinine were enhanced, as is seen in Table 2. Of particular interest is the observation

that in each experiment the K for Rb^{86} rose more markedly than the K for creatinine.

Changes in temperature

As was reported earlier,[10] an elevation in temperature from 28° to 38 °C caused an increase in the Rb^{86} flux. When the migrations of Rb^{86} and creatinine were measured simultaneously, the fluxes of both solutes were enhanced by a 10° rise in temperature (Table 3). In contrast to the results obtained with 5HT (Table 2), the temperature elevation led to a greater percentage increase in the permeability constant for creatinine than in the K for Rb^{86}.

TABLE 2. INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE SIMULTANEOUS MOVEMENTS OF Rb^{86} AND OF CREATININE IN TWO EXPERIMENTS

	K (cm min^{-1} × 10^3)		
	Control*	5HT* (55 μmoles/l.)	%Δ
Rb^{86}	33	49	+48
Creatinine	25	32	+28
Rb^{86}	28	50	+76
Creatinine	23	33	+44

* Each datum is the average value of K obtained from two consecutive periods of observation.

TABLE 3. INFLUENCE OF TEMPERATURE ON THE SIMULTANEOUS MOVEMENTS OF Rb^{86} AND CREATININE IN TWO EXPERIMENTS

	K (cm min^{-1} × 10^3)				
	28 °C		38 °C		
Period no. →	1	2	3	4	%Δ*
Rb^{86}	32	32	45	46	+41
Creatinine	18	17	27	28	+59
Rb^{86}	39	40	50	58	+25
Creatinine	15	16	26	28	+62

* Each percentage change was calculated from the values in periods 2 and 3.

DISCUSSION

Fig. 1 demonstrates that histamine produced a rise in the permeability of the isolated rabbit mesentery and that this rise occurred as soon as the drug was introduced. A new level of permeability was reached in from 5 to 10 min and was sustained thereafter. The other drugs tested in this system also elicited prompt and sustained responses, as did imposed changes in the environmental temperature. Thus equations

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(2) and (3) describe the rubidium flux equally well during control and experimental periods, the only difference being in the magnitude of the permeability coefficient K. That the drug responses in terms of creatinine permeability were also prompt and sustained is not so well established, but is implicit in demonstrations that the creatinine permeability coefficient remained constant for at least two consecutive 45-min periods following exposure to 5HT (Table 2) and following a 10°-rise in temperature (Table 3).

Do these data suggest a mechanism or mechanisms for the transport process? Because neither a hydrostatic pressure gradient nor an osmotic pressure difference was permitted across the membrane, no movement of water was expected and none was observed. Whatever bioelectric potential fields exist between the inside and outside of mesothelial cells, there is no evidence for the existence of a net potential difference between the two outside surfaces of the mesentery, and none would be anticipated in view of its complete bilateral symmetry in terms of both structure and embryogenesis. These considerations restrict the number of possible modes of solute transport.

If either rubidium or creatinine, or both, diffuse passively through either the cells or pores of the mesentery, one presumes that the flux data should be consistent with Fick's diffusion equation for a one-dimensional diffusion process. The relationship between Fick's law and the common empirical equation for membrane permeability (equation 1) is not obvious. Of course the latter is readily inferred from the former if the membrane is infinitesimally thin or if the concentrations (activities) of solute are not allowed to change on the two sides of the barrier. Obviously neither of these conditions was realized in the present system. As long as a steady state is not maintained at the two outside surfaces, the activity gradient along which diffusion occurs cannot be strictly linear within even a homogeneous membrane.

However, Northrup and Anson[16] and McBain and Liu[17] found equation (1) to be satisfactory for describing diffusional fluxes through non-living membranes much thicker than mesentery (sintered glass and alundum), even when the source concentration was allowed to fall and the sink concentration to rise. On theoretical grounds, Barnes[18] has validated these approximations. Thus, if the membrane is thin enough, and the change in sink (or source) concentration is slow enough, equation (1) provides an adequate description of the changing flux. This is equivalent to saying that, as the sink concentration rises, the system promptly approaches a new steady state so that the spacial concentration gradient within the membrane remains sensibly linear, even while its slope gradually falls toward zero (quasi-stationary state). In a multiphase membrane consisting of several layers through which solutes travel in series, the gradient within each phase behaves as though it remains linear.In a membrane such as mesentery, measured values of the permeability coefficient K represent a compromise among the true values for K of the various phases. The form of the compromise for a two-phase system has been derived by Jacobs.[19] The linearity of data like those in Fig. 1 is evidence that the necessary conditions for these approximations of Fick's law have been realized in the present system. Thus, these flux data are consistent, at least qualitatively, with simple passive diffusion as the sole mode of transport.

The present results do not prove that penetration of mesentery by these solutes was due to passive diffusion, and they do not indicate the mechanism or mechanisms by

which drugs modified the permeability constants. As demonstrated here, the permeability to Rb^{86} cations was enhanced by a rise in temperature and by the addition of histamine or of 5-hydroxytryptamine (5HT). Sometimes acetylcholine produced a similar effect, but a response to this substance was not seen consistently. A rise in permeability was also noted in the presence of epinephrine, at least in those instances in which auto-oxidation of the drug did not occur rapidly.

Even more puzzling is the demonstration that environmental factors were able to influence differentially the membrane permeability to two different solutes. Like rubidium, creatinine moved faster under the influence of a 10°-rise in temperature and after the introduction of 5HT. The drug, however, was relatively more effective in enhancing the permeability to rubidium, while a temperature rise produced a relatively greater elevation in the permeability to creatinine. Consequently, one must conclude that 5HT and a rise in temperature operated by different mechanisms to enhance the permeability of mesentery. In other words, if one of these stimuli was active by altering the number, diameter, or length of pores through the mesentery, an entirely different mechanism must be postulated for the other.

An analysis of the environmental factors which influence differentially the permeability to two unlike solutes is expected to clarify the mechanism or mechanisms of membrane transport. At the present time it is premature to speculate about the role of the mesothelial cell in this process, except to note that these cells and perhaps the connective tissue cells must play a vital role, since none of these drug effects can be duplicated with non-living cellulose membranes. Although the specific procedures described in this report are somewhat awkward, these results serve at least to demonstrate the feasibility and potential usefulness of studying the simultaneous penetration of two solutes. With a more convenient double-isotope technique that has now been developed in this laboratory, more extensive studies of this nature will be reported later.

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1 : 1 : 3-TRICYANO-2-AMINO-1-PROPENE (U-9189), A NEW UNCOUPLING AGENT OF OXIDATIVE PHOSPHORYLATION*

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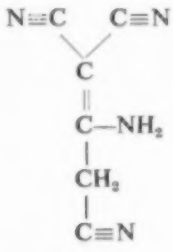
Abstract—1 : 1 : 3-Tricyano-2-amino-1-propene (U-9189) has been shown to uncouple phosphorylation associated with the oxidation of various substrates by rabbit liver mitochondria. It was similar to 2 : 4-dinitrophenol on the basis of its ability to stimulate oxidation in media deficient in inorganic phosphate or phosphate acceptors, to stimulate adenosine triphosphatase activity, or to prevent mitochondrial swelling. Although U-9189 contains both the nitrile and enamine functions, uncoupling was not found to be a general property of either class of compounds.

THE characterization of 1 : 1 : 3-tricyano-2-amino-1-propene (U-9198)† as a component of aqueous solutions of malononitrile recently has been described.[1, 2]



(U-9189)

Preliminary toxicological studies showed that U-9189 administered orally or intraperitoneally to rats produced a rapid and marked hyperthermia.[3] Since DNP also produces a similar hyperthermia, it was of interest to determine whether U-9189 would affect the oxidative–phosphorylation mechanism of isolated mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated by the method of Schneider[4] from livers of New Zealand albino rabbits (from 3 to 5 kg), unselected for sex, or from livers of male Sprague–

* Presented in part at the 138th Meeting of the American Chemical Society, New York, 12 September, 1960.

† Abbreviations used: U-9189: 1 : 1 : 3-tricyano-2-amino-1-propene; ATP: adenosine triphosphate; ATPase: adenosine triphosphatase; DNP: 2 : 4-dinitrophenol; Tris: tris-(hydroxymethyl) aminomethane; RNA: ribonucleic acid.

Dawley (Stoner) rats (from 250 to 400 g). All animals were fed *ad libitum*. For measurement of P/O ratios or oxidation of substrates in deficient media, livers were homogenized in 0·25 M sucrose in a Waring blender for 5 sec at high speed and 10 sec at low speed. For measurement of ATPase activity, livers were homogenized in 0·25 M sucrose with a Potter–Elvehjem type homogenizer with smooth glass tube and Kel-F pestle. For mitochondrial-swelling studies, homogenates were prepared as for the ATPase experiments except that 0·33 M sucrose was used as a homogenizing and suspending medium.

P/O measurements were carried out at 30 °C by the method of Hunter.[5] ATPase measurements were made at 30 °C by the method of Lardy and Wellman.[6] Mitochondrial swelling was determined by following the change in absorbance at 520 mμ at 24 °C in a medium containing 0·33 M sucrose and 0·025 M Tris, pH 7·4.[7] Inorganic phosphate was determined by the method of Lowry and Lopez,[8] and protein was measured by a modification[9] of the procedure of Lowry *et al.*[10]

RESULTS

Uncoupling of oxidative phosphorylation

The curves illustrated in Fig. 1 show that U-9189 was an uncoupling agent of mitochondrial oxidative phosphorylation. Approximately 50 per cent uncoupling was

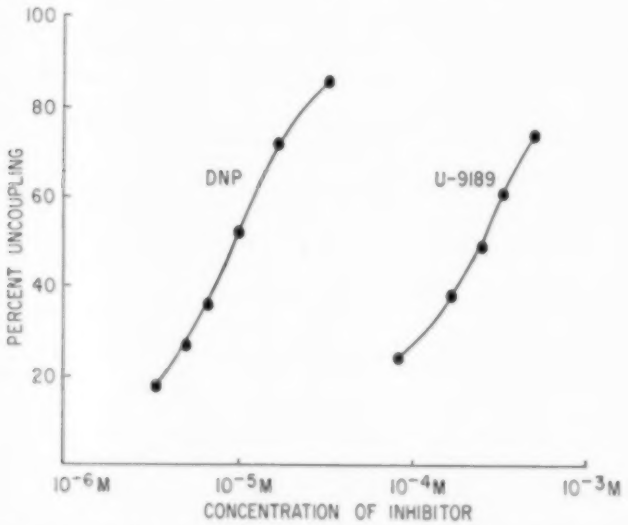


FIG. 1. Uncoupling of oxidative phosphorylation by DNP and U-9189. Complete system[5] plus malonate with α-ketoglutarate as substrate and rabbit liver mitochondria.

produced by $2{\cdot}5\times10^{-4}$ M U-9189 and 1×10^{-5} M DNP. Although DNP was twenty-five times more effective than U-9189 in decreasing P/O ratios, the shapes and slopes of the curves were similar. This order of activity places U-9189 in a category with other interesting uncoupling agents such as thiopentone, chlorotetracycline, 2 : 4-dichlorophenoxyacetic acid, etc.[5]

The uncoupling observed in the oxidation of α-ketoglutarate was not a specific effect related to this substrate. When concentrations of U-9189 and DNP which

produced equivalent uncoupling with α-ketoglutarate were tested with other substrates, similar results were obtained (Table 1). These data suggested that U-9189 might be a DNP-like uncoupling agent, which differed only in the concentration required to produce an equivalent effect.

TABLE 1. UNCOUPLING BY U-9189 AND DNP WITH DIFFERENT SUBSTRATES

Substrate	ΔO (μA/mg prot.)	ΔP (μM)	P : O	% uncoupling
α-Ketoglutarate	0·565	23·9	2·7	—
α-Ketoglutarate + U-9189	0·564	12·1	1·4	50
α-Ketoglutarate	0·523	26·3	2·9	—
α-Ketoglutarate + DNP	0·518	10·5	1·2	59
L-Glutamate	0·765	30·7	2·4	—
L-Glutamate + U-9189	0·776	17·3	1·3	44
L-Glutamate	0·675	31·1	2·2	—
L-Glutamate + DNP	0·681	16·2	1·2	48
Succinate	1·49	17·4	1·3	—
Succinate + U-9189	1·03	5·58	·6	55
Succinate	1·78	25·6	1·4	—
Succinate + DNP	1·28	7·51	·6	61
L-Malate + pyruvate	1·18	28·6	1·9	—
L-Malate + pyruvate + U-9189	1·16	20·4	1·3	32
L-Malate + pyruvate	1·23	23·3	2·0	—
L-Malate + pyruvate + DNP	1·13	11·8	1·2	43

Complete system[5] plus malonate with rabbit liver mitochondria. Malonate omitted in flasks with succinate. [U-9189] = $2·5 \times 10^{-4}$ M, [DNP] = 10^{-5} M.

TABLE 2. STIMULATION OF LIVER MITOCHONDRIA OXIDATION BY U-9189 AND DNP IN PHOSPHATE-DEFICIENT MEDIUM

Enzyme source	Substrate	ΔO (μA/mg prot.)	% stimulation
Rabbit	α-ketoglutarate	0·127	—
Rabbit	α-ketoglutarate + U-9189	0·303	139
Rabbit	α-ketoglutarate + DNP	0·293	131
Rat	α-ketoglutarate	0·060	—
Rat	α-ketoglutarate + U-9189	0·125	108
Rat	α-ketoglutarate + DNP	0·193	222

Complete system[5] plus malonate; phosphate replaced by 60 μM Tris Buffer. [U-9189] = $2·5 \times 10^{-4}$ M, [DNP] = 10^{-5} M.

Stimulation of mitochondrial oxidation in deficient media

In an attempt to characterize further the action of U-9189, it was compared with DNP for its effect on oxidation of substrate by mitochondria in media deficient in inorganic phosphate or phosphate acceptor. In these experiments both U-9189 and DNP were tested at concentrations which produced equivalent uncoupling (50 per cent). Table 2 shows that when inorganic phosphate was omitted from the medium, the addition of either U-9189 or DNP elicited an increased rate of oxidation. Equal stimulation of α-ketoglutarate oxidation by rabbit liver mitochondria was produced

by $2{\cdot}5\times10^{-4}$ M U-9189 and 1×10^{-5} M DNP. In the case of rat liver mitochondria, the effect observed with DNP was much greater than that obtained with U-9189.

When the system was made deficient in phosphate acceptor by omission of ATP, equivalent stimulation of oxidation was produced by the above mentioned concentrations of U-9189 and DNP using mitochondria from either rabbit or rat liver, with either α-ketoglutarate or L-glutamate as substrate (Table 3). In the L-glutamate system essentially maximal stimulation was obtained with three times the concentrations of U-9189 or DNP which produced 50 per cent uncoupling.

TABLE 3. STIMULATION OF LIVER MITOCHONDRIA OXIDATION BY U-9189 AND DNP IN ATP-DEFICIENT MEDIUM*

Enzyme source	Substrate	Molarity uncoupler	ΔO (μA/mg prot.)	% stimulation
Rabbit	α-ketoglutarate		0·124	—
Rabbit	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	0·158	28
Rabbit	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	0·151	22
Rat	α-ketoglutarate		0·544	—
Rat	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	0·737	36
Rat	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	0·762	40
Rabbit	L-glutamate†		0·182	—
Rabbit	L-glutamate† + U-9189	$2{\cdot}5\times10^{-4}$	0·232	27
Rabbit	L-glutamate† + U-9189	$7{\cdot}5\times10^{-4}$	0·314	72
Rabbit	L-glutamate† + U-9189	$1{\cdot}5\times10^{-3}$	0·256	41
Rabbit	L-glutamate† + DNP	$1{\cdot}0\times10^{-5}$	0·232	28
Rabbit	L-glutamate† + DNP	$3{\cdot}0\times10^{-5}$	0·339	86
Rabbit	L-glutamate† + DNP	$6{\cdot}0\times10^{-5}$	0·356	96

* Complete system[5] plus malonate; ATP omitted.
† Malonate omitted.

TABLE 4. STIMULATION OF LIVER MITOCHONDRIA OXIDATION BY U-9189 AND DNP IN HEXOKINASE : GLUCOSE-DEFICIENT MEDIUM

Enzyme source	Substrate	Molarity uncoupler	Malonate	ΔO (μA/ml prot.)	% stimulation
Rabbit	α-ketoglutarate	—	+	0·329	—
Rabbit	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	+	0·412	25
Rabbit	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	+	0·473	44
Rabbit	α-ketoglutarate	—	—	0·377	—
Rabbit	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	—	1·015	169
Rabbit	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	—	1·063	182
Rat	α-ketoglutarate	—	+	0·589	—
Rat	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	+	0·863	47
Rat	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	+	0·904	53
Rabbit	L-glutamate	—	—	0·810	—
Rabbit	L-glutamate + U-9189	$2{\cdot}5\times10^{-4}$	—	1·315	62
Rabbit	L-glutamate + U-9189	$7{\cdot}5\times10^{-4}$	—	1·829	126
Rabbit	L-glutamate + U-9189	$1{\cdot}5\times10^{-3}$	—	1·778	120
Rabbit	L-glutamate + DNP	$1{\cdot}0\times10^{-5}$	—	1·331	64
Rabbit	L-glutamate + DNP	$3{\cdot}0\times10^{-5}$	—	1·885	133
Rabbit	L-glutamate + DNP	$6{\cdot}0\times10^{-5}$	—	1·885	133

Complete system[5] minus glucose and hexokinase.

When the hexokinase–glucose coupling system was omitted from the medium, again U-9189 and DNP appeared to have comparable effects (Table 4) on rabbit and rat liver mitochondria. This conclusion is reinforced by the finding that, at given concentrations of U-9189 and DNP with α-ketoglutarate as substrate, comparable effects were noted in the presence and absence of malonate. As was the case with ATP omission, maximal effect was achieved with three times the concentrations which produced 50 per cent uncoupling.

Stimulation of ATPase activity

Another well-known effect of DNP is its stimulation of mitochondrial ATPase activity. ATPase activity of rabbit or rat liver mitochondria was stimulated by both U-9189 and DNP as shown in Table 5. Although the endogenous ATPase activity of

TABLE 5. STIMULATION OF LIVER MITOCHONDRIAL ATPASE ACTIVITY BY U-9189 AND DNP

Addition	Inorganic phosphate formed (μmoles/10 min per mg protein)	
	Rat	Rabbit
None	0·14	0·57
U-9189, 10^{-3} M	1·53	0·95
U-9189, 10^{-4} M	0·58	0·70
U-9189, 10^{-5} M	0·26	0·65
DNP, 10^{-4} M	1·92	1·19
DNP, 10^{-5} M	1·09	0·80
DNP, 10^{-6} M	0·28	0·66

Reaction mixture contained per ml: 6 μmoles of ATP, 10 μmoles of Tris, 75 μmoles of KCl, and 0·3 ml of mitochondria in 0·25 M sucrose, pH 7·4, 30°C.

rabbit liver mitochondria was always quite high, it is clear that U-9189 treatment enhanced this activity. DNP would be estimated as about twenty-five times more active than U-9189.

Prevention of mitochondrial swelling

Another basis for comparison of uncoupling agents is their effect on mitochondrial swelling. As shown in Fig. 2, U-9189 was effective in preventing swelling induced in rat mitochondria either by 2×10^{-3} M inorganic phosphate or by 10^{-5} M L-thyroxine, as well as spontaneous swelling (induced by Tris buffer). Analogous results were obtained with rabbit liver mitochondria (Fig. 3). Also shown here is the effect of a low concentration of DNP (10^{-5} M). Again DNP was about twenty-five times more active than U-9189.

Effect of other nitriles on oxidative phosphorylation

In Table 6 are listed twelve mononitriles and six dinitriles which were tested for their ability to uncouple oxidative phosphorylation. All compounds were tested at a concentration of 10^{-3} M with a system containing rabbit liver mitochondria and α-ketoglutarate as substrate in the presence of malonate. In no case was there any

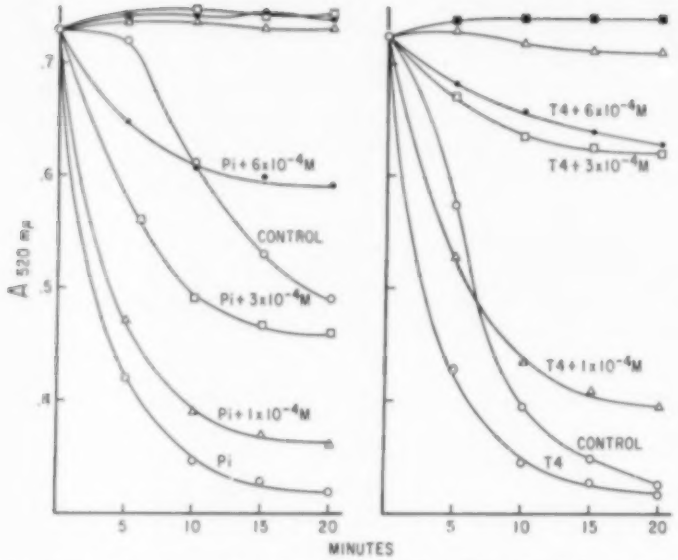


FIG. 2. Inhibition of phosphate- and L-thyroxine-induced swelling of rat liver mitochondria by U-9189. Medium: 0·33 M sucrose, 0·02 M Tris, and 0·002 M phosphate (Pi) or 10^{-5} M. L-Thyroxine (T_4), pH 7·4, 24°C. Unlabeled curves represent controls corresponding to labeled curves (medium plus inhibitor but minus swelling agent).

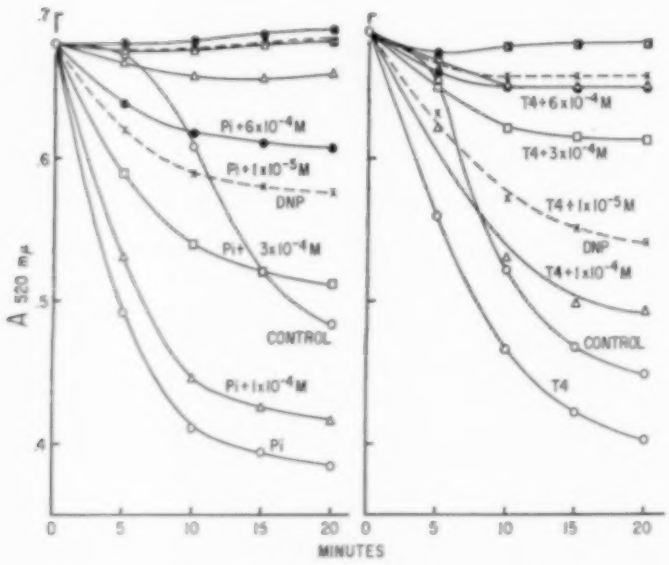


FIG. 3. Inhibition of phosphate- and L-thyroxine-induced swelling of rabbit liver mitochondria by U-9189. Medium: 0·33 M sucrose, 0·02 M Tris, and 0·002 M phosphate (Pi) or 10^{-5} M L-thyroxine (T_4), pH 7·4, 24°C. Unlabeled curves represent controls corresponding to labeled curves (medium plus inhibitor but minus swelling agent).

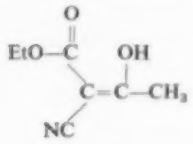
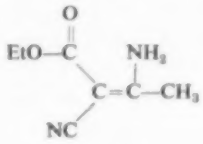
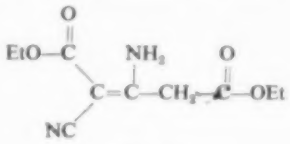
significant effect either on oxidation or phosphorylation. These results were somewhat surprising inasmuch as three of the compounds tested (U-13,758, U-14,509, U-13,761) bear rather close structural similarities to U-9189. Thus, uncoupling would not appear to be a general property of nitriles or enamines.

DISCUSSION

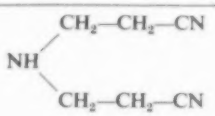
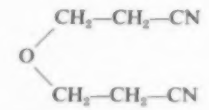
From the data presented in this paper it is clear that U-9189 is a DNP-like uncoupling agent, differing from DNP only quantitatively in effect. In addition, both agents can produce hyperthermia in experimental animals. Since the hyperthermia induced by DNP is considered to be a peripheral effect by some investigators and a central effect by others,[11] certain additional activities of U-9189 should be considered

TABLE 6. NITRILES HAVING NO EFFECT ON OXIDATIVE PHOSPHORYLATION

Monoitriles

$CH_3—CN$	$(CH_3)_2—CH—CN$	$CH_2OH—CH_2—CN$
$CH_3—CH_2—CN$	$CH_3—(CH_2)_3—CN$	$(CH_3)\ (CH_2OH)—CH—CN$
$CH_3—(CH_2)_2—CN$	$HO_2C—CH_2—CN$	$(CH_3)_2—CH—O—(CH_2)_2—CN$
		
U-13,761*	U-14,508*	U-13,758*

Dinitriles

$NC—CH_2—CN$	
$NC—(CH_2)_2—CN$	
$NC—(CH_2)_3—CN$	
$NC—(CH_2)_4—CN$	

* These compounds were synthesized by Dr. G. A. Youngdale, The Upjohn Company.

in this regard. Grenell and Hydén[12] recently demonstrated that U-9189 increased concentrations of protein and RNA in neurons of the rabbit with a concomitant decrease of these components in glial cells. This balance between the two cell populations may explain why we were unable to observe any gross change in total composition of various areas of the brain (unpublished observations). Concentrations of protein and RNA of single Deiter cells were as much as 25 per cent greater than those found in the controls, and the RNA showed altered base ratios.[12] Also, two enzyme activities were shown to be increased several fold, namely cytochrome oxidase and

succinoxidase. In view of these striking changes induced in the central nervous system by U-9189, it is tempting to speculate that the hyperthermia brought about by U-9189 might also be a result of direct action on the central nervous system. However, U-9189 inhibited succinate oxidation in liver mitochondria at concentrations which produced uncoupling (Table 1). Thus, if the U-9189 hyperthermia is centrally mediated it may be produced by some mechanism other than uncoupling of oxidative phosphorylation.

Recent studies by Ingbar[13] showed that in rats U-9189 induced acute inhibition of the organic-binding of iodine, suppressed the formation of thyroxine, and inhibited the conversion of mono- to di-iodotyrosine. Following prolonged administration, these actions were supplemented by inhibition of the thyroidal iodine-concentrating mechanism. Although U-9189 prevented the mitochondrial swelling induced by L-thyroxine, this effect alone does not explain its antithyroid activity. DNP prevents swelling at much lower concentrations and exerts its effects only on the iodide-concentrating mechanism, while U-9189, less potent in uncoupling and swelling-prevention properties, inhibits both the iodide-concentrating and organic-binding mechanisms.[13, 14]

That U-9189 should possess DNP-like activities is not apparent from its structure. Since uncoupling was shown not to be a general property of either nitriles or enamines (Table 6), the effect would appear to be attributed to the special stereochemical configuration of U-9189, or alternatively to a more highly activated enamine grouping. The wide diversity of activities shown by U-9189 suggests that the active sites affected in liver, thyroid, and brain may be different and that some of these are unaffected by DNP. Because of the similarities to and differences from DNP, U-9189 may prove to be a useful tool for the elucidation of the precise mechanism of oxidative phosphorylation and other tissue function.

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ARYLESTERASE IN BLOOD: IRREVERSIBLE INACTIVATION OF THE PLASMA ENZYME—II

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Abstract—Human plasma arylesterase is readily inactivated by urea or guanidine. This process is not reversed by dilution. Depending on the pH, Ca^{2+} and especially La^{3+} slowed down the denaturation considerably. Inhibitors which usually combine with SH-groups did not protect the enzyme. The interrelationship of the process with the ionization of various groups on the protein is discussed.

PREVIOUS publications indicated that the arylesterase of plasma or serum requires the presence of calcium for its activity.[1–3] Thus, it is inhibited by reagents which bind or procedures which remove calcium. Furthermore, it is extremely sensitive to rare earths;[1, 2] these ions inhibit the hydrolysis of phenyl acetate at 10^{-7} M, some even at a lower concentration. Arylesterase is possibly an SH-enzyme. Its activity is blocked by CMPS,* mercuric chloride, and heavy metals.[1, 2]

Since preliminary experiments showed that arylesterase is sensitive to urea,[2] the study of the inactivation of the enzyme seemed to offer more information about the effect of calcium and inhibitors.

MATERIALS AND METHODS

The source of arylesterase was Fraction IV-1 of human plasma.[2, 4] A stock solution, 10 mg/ml, was made up daily in sodium chloride solution (0·1 M) and stored on ice. If not otherwise indicated, final dilutions of the stock solution of 1 : 5 (2 mg of Fraction IV-1 per ml) were incubated at 0 °C with 3 M urea or 1·5 M guanidine hydrochloride in one of the following buffers: at pH 4·0, 4·8, 5·2, 5·7—0·1 or 0·08 M acetate; at pH 6·1, 6·7—0·02 M maleate; at pH 7·5 and 8·2—0·02 M Tris. When the effect of metal ions was studied, this mixture also contained the corresponding salt in the concentration indicated below. Immediately after mixing the enzyme with urea or guanidine, a sample was withdrawn, diluted finally to 1 : 100 v/v in tris buffer of pH 7·3 (0·025 M). After 5 min of pre-equilibration at 27 °C, it was assayed in a Cary recording spectrophotometer at $\lambda = 2800$ Å with a previously described technique.[2] The substrate was 1×10^{-3} M phenyl acetate. The rate of hydrolysis of the first sample was taken as "100 per cent, 0 time reading". Samples were withdrawn afterwards and assayed at regular intervals. The rates were expressed as fractions of the initial rate of hydrolysis.

* Abbreviations: CMPS = *p*-chloromercuriphenylsulfonic acid; Tris = tris-hydroxymethylaminomethane; EDTA and Ca-EDTA = sequestrene : disodium ethylenediaminetetra-acetic acid and its Ca complex; Chel DP = ethylenediamine di(o-hydroxyphenylacetic acid).

In some other studies, 1 mg of Fraction IV-1 per ml was incubated with 5 M urea, and a 1 : 20 v/v final dilution was used at pH 7·2 in the absorption cells of the spectrophotometer. Urea did not inhibit significantly at 0·25 M concentration.

RESULTS

Effect of urea

The inactivation of arylesterase by 3 M urea at pH 7·5 is shown in Fig. 1. Here the relative rate of hydrolysis of the substrate is plotted against time in the presence

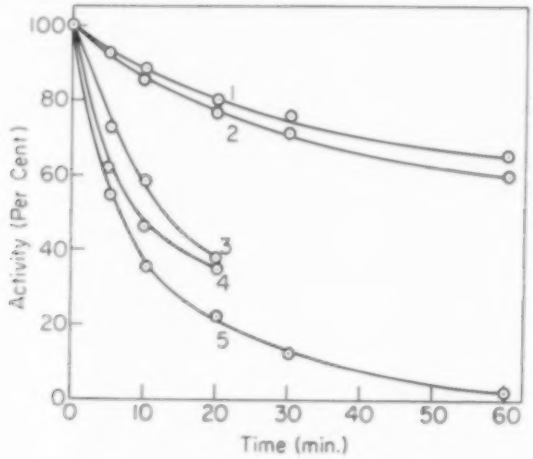

Fig. 1. Inactivation of arylesterase in 3 M urea.
(1) 1×10^{-3} M $LaCl_3$.
(2) 3×10^{-2} M $CaCl_2$.
(3) 1×10^{-2} M $MgCl_2$.
(4) 1×10^{-3} M $HgCl_2$.
(5) Control without added metal ions.

and absence of various agents. The addition of calcium chloride ($CaCl_2$), 3×10^{-2} M, or lanthanum chloride ($LaCl_3$), 1×10^{-3} M, slowed down this inactivation considerably. (The concentration of lanthanum chloride at the final dilution was 1×10^{-5} M, sufficient to have fully inhibited the arylesterase. To avoid this, the solution in the spectrophotometer contained 2×10^{-3} M Ca-EDTA to bind the La^{3+} present. Because La^{3+} has a much higher affinity ($\log K = 15{\cdot}4$) for EDTA than does Ca^{2+} ($\log K = 10{\cdot}6$), it easily replaces calcium in the complex.) Under these conditions, 3 M urea in 1 hr just about completely inactivated the enzyme, while from 60 to 65 per cent of the activity was preserved in the presence of calcium or lanthanum. When, instead of $LaCl_3$, another inhibitor, mercuric chloride ($HgCl_2$), 10^{-3} M, was used, only a fraction of the activity survived (see Fig. 1). (The 1×10^{-5} M final concentration of mercuric chloride also would have completely inhibited the enzyme; hence, we added glutathione, 2×10^{-4} M, as reactivator.[2] Cysteine could not replace glutathione in these experiments, because it is itself a good inhibitor of arylesterase: 85 per cent of the activity was blocked at 1×10^{-4} M concentration by both the D- and L-forms.[5]) A substance, CMPS, which reacts with SH-groups, was even less effective. Magnesium chloride ($MgCl_2$), 1×10^{-2} M, was as little active as mercuric chloride (see Fig. 1), but the activity of strontium chloride ($SrCl_2$), 1×10^{-2} M,

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approached that of calcium chloride. The activity of calcium chloride was quite insensitive to variations in concentration from 3×10^{-3} M to 1×10^{-1} M.

The effect of lower and higher concentrations of urea was also explored. When 5 M urea was used, no activity was detectable in 5 min, but in the presence of 1×10^{-2} M calcium or 1×10^{-3} M lanthanum, 40 and 50 per cent, respectively, remained after incubation for 10 min.

In some experiments with 5 M urea, Ca-EDTA was replaced by another chelating agent, Chel DP. This compound has a very low affinity for calcium ions (log K = 1·6). Although it binds lanthanum ions, it does not inhibit the enzyme,[2] unlike EDTA, which is a good inhibitor of the hydrolysis of phenyl acetate[1-3] or paraoxon[6] by arylesterase. When the arylesterase was incubated with $LaCl_3$, 1×10^{-3} M, in 5 M urea, and mixed after dilution with Chel DP, 2×10^{-4} M, no hydrolysis of the substrate was seen. But when, in addition to Chel DP, $CaCl_2$, 5×10^{-4} M, was added,

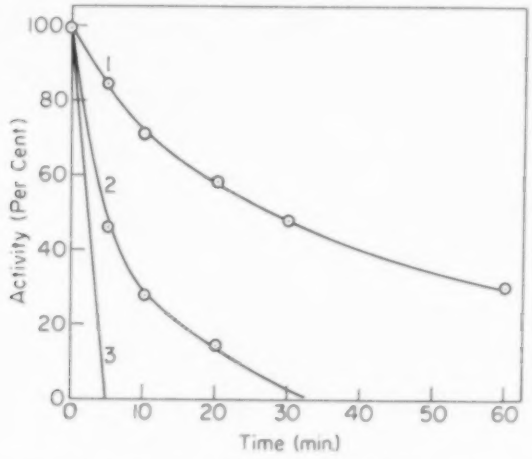

FIG. 2. Inactivation of arylesterase in 1·5 M guanidine.
(1) 1×10^{-3} M $LaCl_3$.
(2) 1×10^{-2} M $CaCl_2$.
(3) Control without added metal ions.

the rate was comparable to the one obtained with Ca-EDTA as chelating agent for $LaCl_3$. Apparently, in addition to the binding of lanthanum ions, the replacement of calcium ions is also necessary here for the reactivation. Lower concentrations of lanthanum chloride, 10^{-4} M, or 2×10^{-5} M, were only slightly less effective against 5 M urea. On the other hand, lowering the urea concentration to 2 M or 1 M greatly lowered the rate of inactivation of the unprotected enzyme. Controls with chelating agents alone gave negative results.

Effect of guanidine

Guanidine, as expected,[7] is a stronger inactivator of arylesterase than is urea. Experiments with guanidine, 1·5 M, and lanthanum chloride, 1×10^{-3} M, at pH 7·5, gave results resembling those with 5 M urea, but calcium protected against guanidine less than against urea (see Fig. 2).

In a control experiment the salt of another rare earth, gadolinium chloride, ($GdCl_3$), behaved much like $LaCl_3$. The effect of lanthanum chloride at lower concentrations of guanidine, 1 M, was apparently more prominent, when compared on the basis of curves like the ones shown in Fig. 2. However, this observation may not be significant, since the rate of inactivation in 1·5 M guanidine is too fast for accurate assay.

Guanidine was also a better inhibitor than urea. About half the activity of arylesterase was inhibited by a 5×10^{-2} M concentration.

Effect of ionic strength

At the pH of the experiments, guanidine is ionized[8] while urea is not. This results in a relatively low ionic strength ($\Gamma/2 = 0{\cdot}03$–$0{\cdot}1$) in the 3 M urea experiments and in a much higher one when guanidine hydrochloride is used. Therefore, the effect of moderate increase in the ionic strength was also investigated. The data revealed that sodium chloride, 0·6 M, did not significantly change the rate of denaturation by 3 M urea. However, the protection of the enzyme by $LaCl_3$, 10^{-3} M, or $CaCl_2$, 10^{-2} M, was more pronounced; only 20 or 26 per cent, respectively, was inactivated in 60 min at pH 7·5.

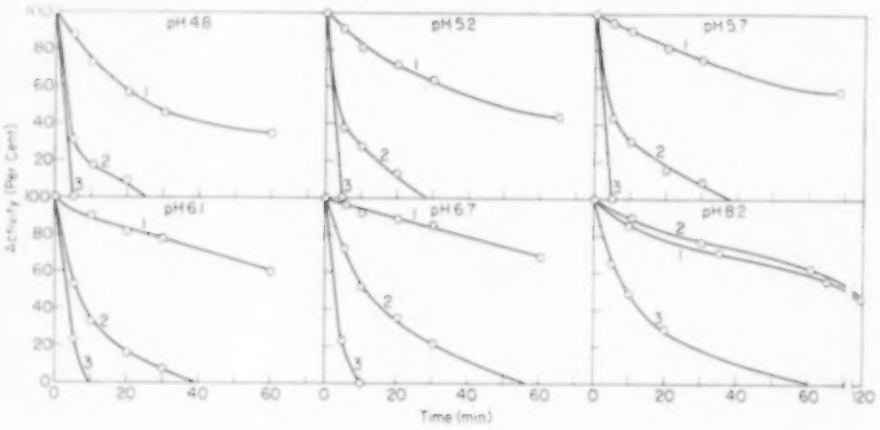

Fig. 3. Inactivation of arylesterase in 3 M urea at various pH values.
(1) 1×10^{-3} M $LaCl_3$.
(2) 1×10^{-2} M $CaCl_2$.
(3) Control without added metal ions.

Effect of pH

This and previous communications[1, 2] have shown that La^{3+} and Ca^{2+} exert a remarkably strong effect on the activity of arylesterase. In order to learn more about the sites of attachment of these ions we decided to study the inactivation of the enzyme at various hydrogen ion concentrations. To eliminate further variations in the experimental conditions, the activity after dilution was assayed at pH 7·3 (±0·1). Lanthanum, which at high concentrations readily forms insoluble hydroxides,[9] remained in solution at pH 8·2 when the solution contained urea.

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In these experiments, the enzyme was incubated with urea in the presence and absence of added ions at pH values ranging from 4·0 to 8·2. At pH 4 the enzyme itself was not stable; more than half of the activity was lost in 40 min, but lanthanum slowed down this inactivation considerably. Also at this pH in 3 M urea or 1·5 M guanidine, the enzyme became inactive very rapidly—within 5 min. Lanthanum and calcium ions seemed to have a negligible effect. Possibly, under these circumstances, all the reactions were too fast to be measured with the present technique.

The experiments at pH values above 4 are shown in Fig. 3. Increasing the pH to 4·8 enhanced the effect of lanthanum while calcium stayed virtually inactive. A great rise in the activity of calcium was seen above pH 6·7. At pH 8·2 the two metals were about equal. The destruction of the unprotected enzyme was much slower at pH 8·2 than at 4·0. Fig. 4 summarizes the results. When the degree of inactivation in 3 M

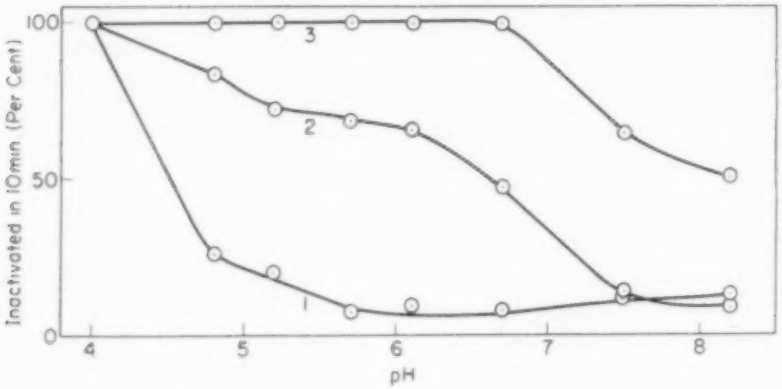

FIG. 4. Effect of pH on the inactivation of arylesterase by 3 M urea during the first 10 min.
(1) 1×10^{-3} M $LaCl_3$.
(2) 1×10^{-2} M $CaCl_2$.
(3) Control without added metal ions.

urea during the first 10 min is compared at various pH values, in the presence of $CaCl_2$, 1×10^{-2} M, or $LaCl_3$, 1×10^{-3} M, the differences between the two salts are easily observed. Thus while $LaCl_3$ became effective above pH 4, the activity of calcium was predominant only well above pH 6.

DISCUSSION

Arylesterase is an enzyme which occurs in the plasma of many members of vertebrata.[10, 11] These experiments showed that urea and guanidine readily destroy the activity of the human arylesterase at 0 °C. Depending on the pH, calcium, lanthanum, and also strontium, slowed down this process, which was not reversed by dilution. The effect of urea and guanidine on the proteins is usually interpreted as the destruction of the secondary structure through breaking of the hydrogen bonds.[12] However, urea is also a competitive inhibitor of a number of enzymes.[13] It has been indicated with some proteins that calcium can protect them against urea.[6, 14–17] On the other hand, acceleration of the process also has been observed.[18] The behavior of the enzymes

toward the denaturing agents varies individually with the pH.[7, 14, 16, 19–22] Since the purpose of these studies was to obtain some information about the groups on the surface of the enzyme which can combine with metal ions, the effect of urea was also studied at various hydrogen ion concentrations. Changes in the pH of the solution are usually accompanied by changes in the ionization on the protein. While studies with a partially purified protein can yield only a limited amount of information, the experiments which showed that the enzyme, after preincubation with either lanthanum chloride or mercuric chloride, needed a complexing agent for restoring the activity, indicated that these metals can combine with a group in the enzyme. The protection by $LaCl_3$ against urea may be attributable to that fact.

We are tempted to correlate the big change in the activity of lanthanum between pH 4·0 and 4·8 with the loss of a proton from a carboxy group on the protein. (Interference of the acetate in the buffer can be disregarded, because adding sodium acetate to the reaction at higher pH levels did not influence the rate. Other considerations make the possibility of interference by the maleic acid buffer unlikely.) The significant change in the effect of calcium chloride between pH 6·2 and 7·5 could be related to a change in the ionization of an imidazole group in the protein or to the change in the α-NH_2 groups of cystine.[23] Indeed, Chervenka[17] indicated that calcium might combine with an imidazole group in α-chymotrypsinogen. Imidazole is considered to be in the active center of a number of enzymes;[23] Mounter *et al.*[24] have made this suggestion for rabbit arylesterase.*

Agents which readily combine with the SH-groups on the protein, such as mercuric ions or CMPS, inhibited the enzyme but did not protect against urea.

It appears to us that the interrelationship of calcium with arylesterase has three facets. Calcium ions accelerate the activity of the enzyme. They are essential for the hydrolysis of the substrate and, finally, they stabilize the structure and protect it against denaturing agents. Lanthanum ions might combine with different groups than do calcium ions; however, they might inhibit the enzyme by replacing calcium ions, as indicated here and in previous experiments.[2]

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* In all of the above considerations the loss of protons by hydrated calcium or lanthanum and the enzyme–metal complexes was not evaluated.

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INFLUENCE OF CHLORPROMAZINE ON DECARBOXYLASES OF AROMATIC AMINO ACIDS

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Abstract—*In vitro* 10^{-4} M chlorpromazine did not influence decarboxylation of 5-hydroxytryptophan (5HTP) and 3 : 4-dihydroxyphenylalanine (dopa) in supernatant of rat brain and kidney. 10^{-3}M chlorpromazine only inhibited dopa decarboxylation in brain supernatant to which no pyridoxal-5′-phosphat (py-5-p) had been added.

Pretreatment of intact rats with high doses of chlorpromazine had no significant effect on decarboxylation of dopa or 5HTP in total homogenates or supernatant of brain.

In supernatant of kidney of rats pretreated with chlorpromazine there was, however, a 30–70 per cent rise in decarboxylation of dopa and 5HTP, provided that no py-5-p was added to the incubation medium.

The results are discussed with regard to the effects of chlorpromazine on the metabolism of py-5-p and aromatic monoamines.

In rat brain chlorpromazine counteracts the following changes in monoamine metabolism:[1]

(a) Release of 5-hydroxytryptamine (5HT), norepinephrine, dopamine (DA) caused by reserpine or benzoquinolizine derivatives.

(b) accumulation of norepinephrine and 5HT due to inhibition of monoamine oxidase (MAO) by iproniazid (N_2-*iso*propyl-*iso*nicotinic acid hydrazide, Marsilid*), isocarboxazid (N_2-benzyl-5′-methyl-3′ isoxazolylcarbonyl hydrazine, Marplan*).

(c) Increase of monoamines following administration of the monoamine precursor 5-hydroxytryptophan (5HTP).

The last two findings would be explained if it could be shown that chlorpromazine inhibits the decarboxylases responsible for the formation of aromatic monoamines. Inhibition of 5HTP decarboxylase in the kidney of chlorpromazine-pretreated rats has in fact been reported,[2] but in these experiments the newly formed 5HT was assayed by the isolated uterus of the oestrous rat. In biological tests of this kind, chlorpromazine might antagonize the pharmacological effect of 5HT.[3, 4] An antagonistic effect of chlorpromazine on 5HT has been observed in other isolated organs, e.g. the colon.[5] The effect of chlorpromazine on the decarboxylases of non-aromatic amino acids has not been clarified. Concerning glutamic acid decarboxylase of brain no effect[6, 7] as well as inhibition[8] was found *in vitro* and *in vivo*.

In the present study, the influence of chlorpromazine on decarboxylation of 5HTP and dopa was investigated *in vitro* and *in vivo* in brain and kidney of rats. Chemical

* Trade name

or manometrical methods were used to determine the reaction products (5HT, DA and CO_2).

METHODS

Non-fasted female Wistar rats of 60–80 g received chlorpromazine i.p.. Untreated rats served as controls. Pools of brains (excluding the medulla oblongata) and of decapsulated kidneys from seven animals were homogenized in 3 or 4 ml per g organ of ice-cold phosphate buffer (0·067 M with pH 8·0 for 5HTP decarboxylase and pH 6·8 for dopa decarboxylase). A Potter–Elvehjem homogenizer (Teflon, clearance 0·004–0·006 in.) was used for brain and a Servall Omni-mixer (16·000 rev/min. for 45 sec) for kidney.

(a) *Enzyme activity in brain*

Dopa- and 5HTP decarboxylase were measured in 3 ml supernatant (29,000 g at 2 °C for 30 min) by the slightly modified procedure of Davis and Awapara.[9] Incubations were carried out with 10 μmoles dopa (with or without 0·377 μmoles pyridoxal-5′-phosphate (py-5-p)) for 30 min, or with 12·5 μmoles 5HTP (with or without 0·038 μmoles py-5-p) for 60 min under N_2. The newly formed DA or 5HT was estimated spectrophotofluorometrically after adsorption of the monoamines on Amberlite CG-50 (final washing with 20 ml 0·67M phosphate buffer, pH 6·8). Non-incubated samples served as blank.

In additional experiments, 5HTP decarboxylase was estimated in total homogenates and in supernatant by spectrophotofluorometric measurement of the newly formed 5HT.[10] Incubations were carried out as above but on two occasions 1 ml each of homogenate or supernatant was used, adding 5·0 μmoles 5HTP, with or without 0·038 μmoles py-5-p.

(b) *Enzyme activity in kidney*

From 1 to 2 ml of supernatant of kidney homogenate were incubated in Warburg manometers with 25 μmoles dopa (with or without 0·18 μmoles py-5-p) or with 25 μmoles 5HTP (with or without 0·038 μmoles py-5-p). The final volume was 2·4 ml, with a pH the same as in the brain experiments. Incubation was carried out at 37·5 °C under N_2. It was stopped after 15 and 60 min, respectively, by tipping in 0·1 ml 20% H_2SO_4 from the second side arm. Calculation of enzyme activity from the initial velocity of CO_2-formation was done. Incubations without substrate served as blanks.

In some *in vitro* experiments chlorpromazine was added before incubation to aliquots of supernatant of brain and kidney of normal rats.

RESULTS AND DISCUSSION

Results of *in vitro* experiments are summarized in Table 1. Chlorpromazine in concentrations as high as 10^{-4} M does not inhibit decarboxylation of 5HTP or dopa significantly in enzyme preparations of rat brain and kidney ($P>0·05$). 10^{-3}M chlorpromazine only inhibits dopa decarboxylation in brain supernatant in the absence of py-5-p. This finding is probable without biological significance.

Results obtained in *intact animals* pretreated with chlorpromazine are shown in Table 2. Single or repeated doses of 20 or 25 mg/kg chlorpromazine i.p. have no significant effect on decarboxylation of 5HTP or dopa in *brain* ($P>0·05$), whether or not py-5-p is added to the incubation medium. In kidney there is a significant increase

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TABLE 1. INFLUENCE OF CHLORPROMAZINE ON DECARBOXYLATION OF AROMATIC AMINO ACIDS *in vitro*

Supernatant of brain and kidney homogenates was used as enzyme source. The experimental conditions corresponded to the *in vivo* experiments. Absolute activity of the enzyme see Table 2(a). The figures represent mean values ± standard error in per cent of controls. Number of estimations in parenthesis.

Addition (final concentration)	Brain		Kidney	
	Without pyridoxal-5′-phosphate	With pyridoxal-5′-phosphate	Without pyridoxal-5′-phosphate	With pyridoxal-5′-phosphate
	Substrate dopa			
none	100 ± 3 (2)	100 ± 1 (2)	100 ± 9 (3)	100 ± 1 (2)
chlorpromazine 10^{-3}M	69 ± 4 (2)	95 ± 2 (2)	102 ± 4 (3)	85 ± 6 (2)
chlorpromazine 10^{-4}M	99 (1)	102 (1)	98 ± 6 (3)	92 ± 2 (2)
	Substrate 5HTP			
none	100 (1)	100 (1)	100 ± 11 (3)	100 ± 5 (4)
chlorpromazine 10^{-3}M	106 ± 11 (2)	113 ± 2 (2)	84 ± 9 (3)	91 ± 2 (4)
chlorpromazine 10^{-4}M	—	—	88 ± 4 (3)	98 ± 2 (4)

TABLE 2. DECARBOXYLATION OF DOPA AND 5HTP BY BRAIN AND KIDNEY OF CHLORPROMAZINE-PRETREATED RATS

The figures express mean values ± standard error of enzyme activity in per cent of controls. The figures are derived from two to five experiments with seven animals each.
The absolute activity of control groups is reported in Table 2(a).

Pretreatment prior to decapitation	Brain				Kidney	
	Total homogenate		Supernatant		Supernatant	
	Without pyridoxal-5′-phosphate	With pyridoxal-5′-phosphate	Without pyridoxal-5′-phosphate	With pyridoxal-5′-phosphate	Without pyridoxal-5′-phosphate	With pyridoxal-5′-phosphate
			Substrate dopa			
none	—	—	100 ± 4A	100 ± 1A	100 ± 3	100 ± 1
1 × 20 mg/kg chlorpromazine 1 hr	—	—	100 ± 5A	109 ± 3A	104 ± 3	97 ± 3
1 × 20 mg/kg chlorpromazine 3 hr	—	—	106 ± 4A	96 ± 2A	129 ± 2*	99 ± 3
3 × 25 mg/kg chlorpromazine within 3 days, last dose 16 hr	—	—	101 ± 8A	89 ± 7A	129 ± 1*	97 ± 1
			Substrate 5HTP			
none	100 ± 2B	100 ± 3B	100 ± 1B 100 ± 4A	100 ± 2B 100 ± 3A	100 ± 1	100 ± 1
1 × 20 mg/kg chlorpromazine 1 hr	103 ± 2B	97 ± 1B	105 ± 3B 100 ± 4A	106 ± 5B 93 ± 8A	141 ± 17	94 ± 12
1 × 20 mg/kg chlorpromazine 3 hr	102 ± 4B	99 ± 2B	114 ± 3B 115 ± 5A	107 ± 1B 109 ± 3A	170 ± 4*	108 ± 2
3 × 25 mg/kg chlorpromazine within 3 days, last dose 16 hr	—	—	109 ± 1B	85 ± 4B	170 ± 6§	117 ± 5

* $P = < 0.01$ in comparison to controls (Student's test).
A = method according Davis and Awapara[9] with cation exchanger and photometry.
B = spectrophotofluorometric determination of 5HT according to Bogdanski *et al.*[10]
§ two experiments only.

in decarboxylation of both amino acids ($P<0{\cdot}01$). This marked increase of enzyme activity can be observed regularly in experiments carried out without addition of py-5-p. The results with kidney are in disagreement with a previous observation that the activity of 5HTP decarboxylase in rat kidney fell by 64 per cent after repeated administration of chlorpromazine.[2] This discrepancy can possibly be explained by interference of chlorpromazine in the biological test (see above).

The observation that chlorpromazine activates 5HTP and dopa decarboxylase in kidney is interesting in the following two respects:

(a) The activation takes place only when there is no additional py-5-p in the incubation medium. If optimal amounts of the coenzyme are added, results do not differ from those obtained in control animals (Table 2).

(b) Chlorpromazine-pretreatment increases the decarboxylation less than addition of py-5-p to the incubation medium. Thus, in supernatant of chlorpromazine-treated animals the activity of dopa and 5HTP decarboxylase is 129 per cent and 170 per cent, respectively compared with supernatant from untreated controls not supplemented with py-5-p (Table 2). Addition of py-5-p to supernatant of controls, however, stimulates the activity up to 422 per cent and 213 per cent, respectively (Table 2(a)).

Table 2(a). Absolute enzyme activity

Organ	Preparation		Monoamine	μmoles/g_{fr}/hr	pyridoxal-5′-phosphate	number of experiments
brain	total homogenate	B	5HT	0·26 ± 0·006	—	12
			5HT	0·27 ± 0·007	+	12
	supernatant	A	DA	1·40 ± 0·06	—	65
			DA	2·00 ± 0·02	+	64
	supernatant	B	5HT	0·14 ± 0·002	—	45
			5HT	0·16 ± 0·003	+	44
	supernatant	A	5HT	0·21 ± 0·008	—	13
			5HT	0·22 ± 0·006	+	13
kidney	supernatant		DA	23·3 ± 0·7	—	51
			DA	98·3 ± 0·8	+	51
			5HT	3·9 ± 0·02	—	27
			5HT	8·3 ± 0·02	+	26

A = method according to Davis and Awapara[9] with cation exchanger and photometry.
B = spectrophotofluorometric determination of 5HT according to Bogdanski *et al.*[10]

These results suggest that chlorpromazine facilitates the formation of the complex apoenzyme/py-5-p in kidney, possibly by interfering with the metabolism of the coenzyme. It has, indeed, been found that chlorpromazine relieves some symptoms of vitamin B_6-deficiency in rats.[11] Furthermore, there is experimental evidence suggesting that chlorpromazine may interfere with pyridoxal-kinase which catalyses the formation of py-5-p from pyridoxal and adenosine-5′-triphosphate (ATP). *In vitro* the phenothiazine stimulates pyridoxalkinase in rat brain,[12] whereas *in vivo* excessive doses of this drug have an inhibitory action.[6] This divergency may be due to the fact that chlorpromazine either inhibits or stimulates the utilization of ATP depending on the concentration ratio of Mg^{2+}/ATP in the incubation system.[12]

As seen in preliminary experiments the effect of chlorpromazine on decarboxylase activity in rat brain and kidney is not appreciably changed by additional pretreatment with iproniazid, 16 hr before chlorpromazine injection.

Since according to the present experiments chlorpromazine does not inhibit decarboxylation of dopa or 5HTP in rats other mechanisms might be responsible for the interference of the drug with monoamine metabolism as outlined above. For instance, chlorpromazine could act by changing the permeability of membrances for monoamines[1] and amino acids.[14]

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INCREASED ACTIVITY OF ACID PHOSPHATASE AND β-GLUCURONIDASE IN THE LIVER AND SPLEEN OF MICE AFTER INTRAPERITONEAL ADMINISTRATION OF VARIOUS MACROMOLECULAR SUBSTANCES

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Abstract—In the present investigation the macromolecular substances dextran, polyvinylpyrrolidone, Periston and carbon were injected into the peritoneal cavity of mice. The acid phosphatase and β-glucuronidase activities of the liver and spleen were investigated biochemically and histochemically. The localization of the macromolecular materials stored in these organs was investigated histochemically.

Histochemical results. In the liver the macromolecular substances were stored in the cytoplasm of the Kupffer cells, and dextran also in the cytoplasm of the parenchymal cells. In the spleen the macromolecular substances were exclusively stored in the cytoplasm of the reticulum cells.

In the livers of the control animals, activity of the two enzymes was found almost exclusively in the Kupffer cells. In the livers of injected animals, activity of the two enzymes was seen in both the Kupffer and the parenchymal cells. In the spleen, only the reticulum cells surrounding the follicles show acid phosphatase and β-glucuronidase activity.

Biochemical results. The total activity of acid phosphatase and β-glucuronidase in the liver and spleen of mice injected with the specified macromolecular substances was significantly increased compared with control animals which were not injected or which received injections of physiological saline solution. The total activity of both enzymes in both organs showed no variation as a result of fasting or injection with physiological saline solution. A possible correlation is also indicated between the activity of acid phosphatase and of β-glucuronidase per unit weight for both liver and spleen in those mice which were not injected with macromolecular substances. This correlation could, however, not be demonstrated after the injection of the macromolecular substances. As a result of the storage of the macromolecular substances, the weights of the liver and of the spleen rose significantly.

INTRODUCTION

During a previous histochemical investigation, Willighagen[1] observed that the activity of acid phosphatase is sharply increased in macrophages present in chronically inflamed lungs and in the neighbourhood of a degenerating corpus luteum. These macrophages also show a great deal of material deriving from degenerating and necrotic tissue. These observations suggested that the incorporation of macromolecular substances may be correlated with a rise in the activity of acid phosphatase.[2] The possible existence of a relation between storage in cells and a high acid phosphatase activity seemed further strengthened by the high acid phosphatase activity found in reticuloendothelial cells such as those in the liver and spleen, which are known for their capacity to store many materials of microscopic and

macromolecular dimensions in their cytoplasm.[3] In order further to investigate this hypothetical correlation between storage of macromolecular substances and increased acid phosphatase activity, an investigation was made of the activity of acid phosphatase in mouse livers after the mice had been injected with dextran.[4]

Using histochemical methods it was found that after intraperitoneal administration the dextran is incorporated both in the parenchymal cells and in the Kupffer cells of the liver, and that this storage was indeed accompanied by a rise in acid phosphatase activity in the parenchymal cells. An increase in enzyme activity was also observed in liver homogenates.[4] Although it is known that dextran is broken down very slowly in the liver of the animal organism[5–7] and although there are still no indications that the acid phosphatase enzyme complex plays any part in the metabolism of the monomer of dextran (glucose), the possibility that the increase in the acid phosphatase activity is connected with a metabolic digestion of the ingested substance cannot be entirely excluded. This suggested the use of metabolically inert macromolecular substances such as polyvinylpyrrolidone and carbon particles in a subsequent study.[8, 9]

Investigations by de Duve *et al.*[10] showed that in rat liver cells the acid phosphatase enzyme complex is found primarily in lysosomes. In addition to acid phosphatase, lysosomes contain still other hydrolytic enzymes. It seemed to us of interest to determine not only the acid phosphatase activity but also the activity of another enzyme found in lysosomes. Our choice fell on the enzyme β-glucuronidase. In the investigation described in this paper, the activity of acid phosphatase and of β-glucuronidase was studied histochemically and biochemically in the spleen as well as in the liver. Determinations with the biochemical method yielded quantitative data concerning the changes in activities of the two enzymes in tissue homogenates as a result of the storage of the macromolecular materials. These results, however, represent mean values for a variety of cellular elements and extracellular fluids. To translate these results in terms of the structural units of tissue, histochemical methods are used to determine in which of these units the activity is located.

EXPERIMENTAL

Mice of the O_{20} (Amsterdam) strain from 4 to 6 months old, were used. After intraperitoneal administration of the macromolecular substances, the acid phosphatase and β-glucuronidase activity in the liver and spleen were biochemically and histochemically investigated. In addition, the cellular storage sites of the various macromolecular substances was studied histochemically.

Macromolecular substances

(1) *Dextran*, average molecular weight 160,000. The dextran was obtained from Poviet & Co., Amsterdam. From the dextran a 6 per cent sterile solution in 0·9 per cent NaCl was prepared.

(2) *Polyvinylpyrrolidone*, a polymer of vinylpyrrolidone with an average molecular weight of 640,000 (Bayer, Leverkusen). A 6 per cent solution in 0·9 per cent NaCl was prepared.

(3) *Periston*, a polymer of vinylpyrrolidone; average molecular weight 17,000. The Periston was supplied as a 4 per cent solution in 0·9 per cent NaCl (Bayer, Leverkusen).

(4) *Carbon particles*. From an India-ink suspension (Günther Wagner; Hannover, Tusche no. C11/1431a) a 2 per cent suspension of carbon particles in 0·9 per cent

NaCl solution was prepared. The average diameter of the carbon particles was, according to specification, 25 mμ.[11]

Injection scheme

Seven groups of animals were used, each group consisting of seven animals. The animals were killed on the tenth day after the first injection. Acid phosphatase and β-glucuronidase activity was determined in homogenates of liver and spleen. One control group (I) received no treatment. A second control group (II) was fasted for 18 hr before being killed, an ample supply of drinking water being available. The third group (III) was given a daily intraperitoneal injection of 1 ml of 0·9 per cent NaCl solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group IV was given daily intraperitoneal injections of 1 ml of dextran solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group V was given daily injections of polyvinylpyrrolidone solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group VI was given daily injections of 1 ml of Periston for 9 days and fasted for 18 hr before being killed on the tenth day. Group VII was given daily injections of 1 ml of carbon suspension for 5 days and fasted for 18 hr before being killed on the tenth day. (Because the O_{20} mice did not react well to nine carbon injections, only five injections were given, on the tenth, eighth, sixth, fourth and second day before the animal was killed).

Histochemistry

Pieces of liver and spleen tissue were quickly frozen on dry ice and series of sections with a thickness of 10 μ were made in a cryostat. The sections were fixed in an aqueous solution consisting of 10 ml of a 4 per cent aqueous solution of formaldehyde and 10 ml of a 2 per cent aqueous solution of $CaCl_2 \cdot 2H_2O$.

To this fixative 80 ml of a 6 per cent aqueous solution of dextran was added as an osmotic protection.[12] Acid phosphatase activity was demonstrated in these sections with Gomori's method.[13] Histochemical activity of β-glucuronidase was demonstrated with Fishman and Baker's method[14] on cryostat sections and also on formalin fixed frozen sections according to Holt.[15] For the demonstration of dextran, sections were fixed in absolute ethanol and stained with the alcoholic periodic acid Schiff method.[16] We were unable to stain polyvinylpyrrolidone histochemically, but in the haematoxylin-stained sections an accumulation of basophilic substance could be seen in the Kupffer cells. This may indicate the site of the stored polyvinylpyrrolidone. The sections used for the demonstration of the carbon particles were fixed in a 4 per cent aqueous solution of formaldehyde.

Biochemistry

Small quantities of tissue (200 mg) taken from different parts of the liver and from the whole spleen were weighed rapidly on a torsion balance. The tissue was homogenized for 1½ min in a ground-glass Potter–Elvejhem homogenizer in about 20 ml of twice distilled water. After homogenization, more distilled water was added to bring the tissue concentration to 100 mg tissue per 20 ml water. To 10 parts of this solution were added 1 part of a 1 per cent solution of Triton-X-100 (a gift from Rohm and Haas & Co., Philadelphia, U.S.A.) to achieve maximal solubilization of the enzymes and availability of the substrates.[17] The homogenates to which Triton-X-100 had been added were allowed to stand for 30 min at 4 °C.

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Acid phosphatase activity determinations. Acid phosphatase activity at pH 4·8 was determined as reported previously.[4] Disodium β-glycerophosphate was used as substrate in both the histochemical and biochemical experiments. The product contained 2·1 per cent of the α-isomer (determined according to Toal and Phillips[18]).

β-Glucuronidase activity determinations. The activity of the enzyme β-glucuronidase in the homogenate was determined using phenolphthalein-β-D-monoglucuronide as a chromogenic substrate, the liberated phenolphthalein being determined colorimetrically. The substrate phenolphthalein-β-D-monoglucuronide was prepared according to Fishman *et al.*[19] The estimations were carried out as described by Talalay *et al.*[20] at 37 °C and pH 4·5. Each determination was made in triplicate with a single control.

Triton-X-100 in the concentration used was found not to disturb the acid phosphatase and β-glucuronidase determinations. Aliquots of the homogenates were analyzed for total nitrogen by the Kjeldahl method.[21]

RESULTS

Histochemistry

Liver. The dextran was found to be stored in the cytoplasm of the Kupffer and parenchymal cells in the form of small droplets. The parenchymal cells located around the central vein stored more dextran than those lying peripherally. In the sections stained with haematoxylin, a weakly basophilic substance was observed in the cytoplasm of the Kupffer cells of animals which had been injected with polyvinylpyrrolidone. This is probably the location of the polyvinylpyrrolidone storage, since the livers of animals which were not injected with this substance did not show this phenomenon. Carbon particles were found stored in the cytoplasm of the Kupffer cells and not in the cytoplasm of the parenchymal cells. We did, however, observe that compact fields of histiocytic cells were present in the liver which showed signs of intense phagocytosis of carbon.

The livers of the control mice (Groups I, II and III) showed a high degree of acid phosphatase activity in the cytoplasm of the Kupffer cells. The acid phosphatase activity in the cytoplasm of parenchymal cells of the control animals, however, was low. The livers of mice injected with the macromolecular substances show high acid phosphatase activity in the cytoplasm of both the Kupffer cells and the parenchymal cells.

The methods described in the literature for histochemical determination of β-glucuronidase are still far from ideal. Even with the Fisher and Baker method used in this investigation, the localization of the enzyme was not sharp, while the intensity of the histochemical stain was not sufficiently representative of the activity of the enzyme in the tissue slices. In spite of these difficulties, it was clear that in the liver activity of β-glucuronidase is to be found in the same locations as activity of acid phosphatase. For the reasons just mentioned, however, it was impossible to determine accurately whether there was a variation of the activity of β-glucuronidase as a result of the intraperitoneal administration of macromolecular substances. In the mice injected with macromolecular substances the Kupffer cells were swollen and had increased in number.

Spleen. In the spleen the macromolecular materials were present only in the reticulum cells. Most of the macromolecular material was stored in the cytoplasm of the

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reticulum cells surrounding the follicles, while only a few of the reticulum cells of the reaction centres of the follicles had stored. Only the reticulum cells show acid phosphatase and β-glucuronidase activity. After injection with the macromolecular materials the reticulum cells showed a swollen appearance and a high activity of acid phosphatase and β-glucuronidase. Because of the already high activity of these enzymes in the reticulum cells of the control animals, it was impossible to determine whether this activity had risen as a result of the incorporation of the injected substances.

Biochemistry

The results of the acid phosphatase and β-glucuronidase determinations are given in Tables 1 and 2.

In order to compensate for fluctuations in hepatic and splenic weight and for the use of animals of varying body weight, the specific activity of the enzymes acid phosphatase and β-glucuronidase was multiplied by the weight of the intact liver or spleen and divided by the body weight of the animal. This calculation normalized values to give the total enzyme activity in the two organs per g body weight.

TABLE 1. THE AVERAGE QUOTIENTS Q AND THE AVERAGE ENZYME ACTIVITIES OF THE LIVERS OF THE VARIOUS GROUPS OF MICE ($\pm$ s.d.)
(s.d. $= \sqrt{[\Sigma (X - \bar{X})^2/(n-1)]}$)

Group	Q*	Activity†	
		Acid phosphatase	β-Glucuronidase
		pH 4·8	pH 4·5
I. Controls (not fasted)	49·2 ± 2·4	109 ± 17	269 ± 51
II. Controls (fasted)	37·9 ± 2·3	111 ± 16	234 ± 47
III. Controls (0·9% NaCl)	40·1 ± 2·9	128 ± 18	259 ± 52
IV. Dextran, m.w. = 160,000	49·4 ± 2·5	287 ± 69	421 ± 60
V. PVP, m.w. = 640,000	44·4 ± 6·2	275 ± 49	388 ± 48
VI. PVP, m.w. = 17,000	47·9 ± 3·4	261 ± 65	409 ± 114
VII. Carbon ϕ 250 Å	48·5 ± 2·8	224 ± 47	358 ± 59

* $Q = \frac{\text{g(liver)}}{\text{g(mouse)}} \times 1000.$

† Activity $= \frac{\mu\text{g P(liver)}}{\text{g(mouse).hr}}$ or $\frac{\mu\text{g phenolphthalein (liver).}}{\text{g(mouse).hr}}$

Nitrogen determinations in liver and spleen showed that the nitrogen content per 100 mg wet tissue remains practically constant in all seven groups of mice irrespective of treatment.

Table 3 gives the enzyme activity per mg N.

DISCUSSION

It may be concluded from the quotient Q in Tables 1 and 2 that:

(1) As a result of fasting, liver and spleen weights drop.

(2) As a result of the incorporation of macromolecular materials liver and spleen weights rise (compare Group III with Groups IV, V, VI and VII).

Table 2. The average quotients Q and the average enzyme activities of the spleens of various groups of mice ($\pm$ s.d.)

Group	Q*	Activity†	
		Acid phosphatase	β-glucuronidase
		pH 4·8	pH 4·5
I. Controls (not fasted)	2·4 ± 0·3	14·4 ± 2·6	43·8 ± 2·9
II. Controls (fasted)	1·9 ± 0·4	14·6 ± 1·6	43·1 ± 8·6
III. Controls (0·9% NaCl)	2·0 ± 0·5	13·2 ± 1·7	50·6 ± 9·3
IV. Dextran, m.w. = 160,000	6·8 ± 1·3	62·9 ± 12·5	104·0 ± 29·1
V. PVP, m.w. = 640,000	5·0 ± 0·8	45·7 ± 5·7	93·5 ± 11·7
VI. PVP, m.w. = 17,000	3·2 ± 0·5	28·9 ± 4·8	73·1 ± 8·1
VII. Carbon ϕ 250 Å	6·6 ± 1·2	38·0 ± 7·5	138·0 ± 31·6

* $Q = \frac{\text{g(spleen)}}{\text{g(mouse)}} \times 1000.$

† Activity $= \frac{\mu\text{g P(spleen)}}{\text{g(mouse).hr}}$ or $\frac{\mu\text{g phenolphthalein (spleen)}}{\text{g(mouse).hr}}$

Table 3. The average enzyme activities per mg N of liver and spleen, of the various groups of mice ($\pm$ s.d.)

Group	Acid phosphatase activity (μg P/hr per mg N)		β-Glucuronidase activity (μg phenolphthalein/hr per mg N)	
	liver	spleen	liver	spleen
I. Controls (not fasted)	87 ± 13	214 ± 32	216 ± 73	650 ± 90
II. Controls (fasted)	99 ± 14	282 ± 36	208 ± 41	830 ± 128
III. Controls (0·9% NaCl)	107 ± 16	310 ± 34	217 ± 45	925 ± 147
IV. Dextran, m.w. = 160,000	211 ± 47	348 ± 76	309 ± 52	575 ± 121
V. PVP, m.w. = 640,000	231 ± 44	331 ± 47	283 ± 42	677 ± 109
VI. PVP, m.w. = 17,000	199 ± 38	336 ± 60	286 ± 80	851 ± 122
VII. Carbon ϕ 250 Å	155 ± 32	352 ± 39	247 ± 43	832 ± 203

(3) The total acid phosphatase activity and the total β-glucuronidase activity in the liver and spleen do not vary as a result of fasting or injection with 0·9 per cent NaCl solution.

(4) The total acid phosphatase activity and the total β-glucuronidase activity in the liver and spleen rise significantly as a result of the incorporation of the macromolecular substances used (significance for the physiological saline mice of Group III: $P \leqslant 0{\cdot}05$).

The combination of biochemical and histochemical results thus show that a considerable increase in the acid phosphatase activity and β-glucuronidase activity of mouse liver homogenates and spleen homogenates accompanies storage of macromolecular substances in both organs. In the liver the increase in activity of acid phosphatase and β-glucuronidase is localized in the parenchymal and Kupffer cells. In the spleen the increase in activity for both enzymes is localized in the reticulum cells. The elevation in activity of the two enzymes might be visualized as a reflection

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of an increased quantity of enzyme or as an alteration in the properties of the enzyme in the direction of increased catalytic activity, or as a combination of both.

Since the calculated total enzyme activity of acid phosphatase and β-glucuronidase of the whole organ does not change as a result of fasting or injection with physiological saline, it can be excluded that the rise in enzyme activity in the animals injected with the macromolecular substances is a result (or partial result) of a change in the glycogen level or of a stress factor. A direct metabolic effect can also be excluded because the rise in enzyme activity takes place not only with the macromolecular substance dextran, which is largely but not completely metabolically inert, but also with the metabolically inert substances Periston, polyvinylpyrrolidone and carbon.

The rise in enzyme activity is probably not caused by the ingestion process in the cell. This conclusion is based on data reported in a previous publication[4] which indicated that some days after the last injection the acid phosphatase activity is still very high, as well as on the fact that a rise in enzyme activity can be demonstrated by the biochemical determination method only after an injection series lasting at least three days.

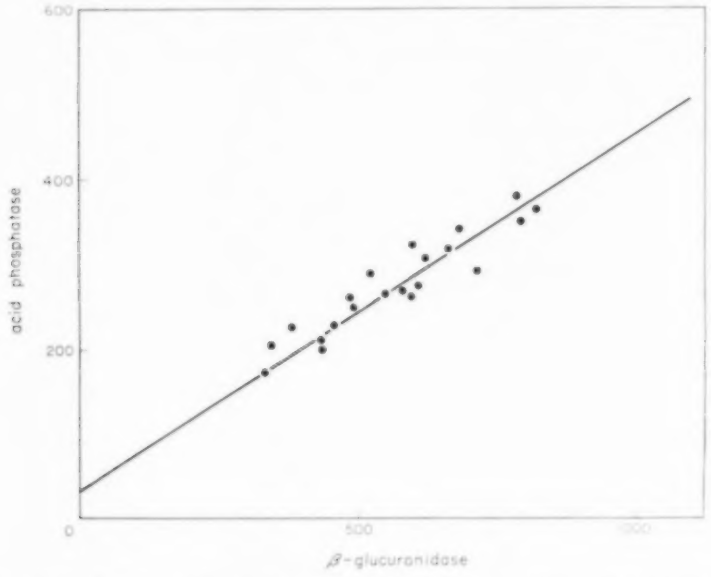

FIG. 1. Correlation between acid phosphatase activity and β-glucuronidase activity per 100 mg wet liver tissue of the first three groups of mice. Acid phosphatase activity: μg P/100 mg wet liver per hr. β-Glucuronidase activity: μg phenolphthalein/100 mg wet liver per hr.

It is therefore probable that our results indicate the existence of a special relationship between acid phosphatase and β-glucuronidase activity and the cellular storage of certain substances. The phenomenon of phagocytosis or pinocytosis followed by storage in different types of cells such as found in rat liver and rat kidney tissue has been related to small cytoplasmic granules.[22, 23] Granules of this type, as far as they have been studied, have been found to be rich in acid phosphatase and β-glucuronidase.[10]

According to various authors, the activity of glucose-6-phosphatase and the activity of fructose-1:6-diphosphatase, enzymes which play an important part in carbohydrate metabolism, are highly dependent upon the nature of the animals nutrition. It has also been shown that as a result of fasting the activity of glucose-6-phosphatase rises, while the activity of fructose-1:6-diphosphatase drops.[24] Since it appears from the present data that the total acid phosphatase activity and the total β-glucuronidase activity do not vary as a result of fasting, neither of these enzymes would seem to play an important role in glycogeen metabolism.

In Fig. 1 for the first three groups of mice the acid phosphatase activity per 100 mg wet liver tissue is plotted for each mouse individually against the β-glucuronidase activity per 100 mg wet liver tissue. In Fig. 2 the same is done for the spleen. The distribution of the points suggests a possible relationship between the acid phosphatase and the β-glucuronidase activities. This correlation is statistically significant as demonstrated with Fisher's χ^2 test ($P \leq 0{\cdot}05$). The lines drawn in the figures are calculated from the position of the points according to Youden.[25] Enzyme determinations in liver from other, unpublished investigations with O_{20} mice also indicate a possible correlation between the activity of acid phosphatase and β-glucuronidase.

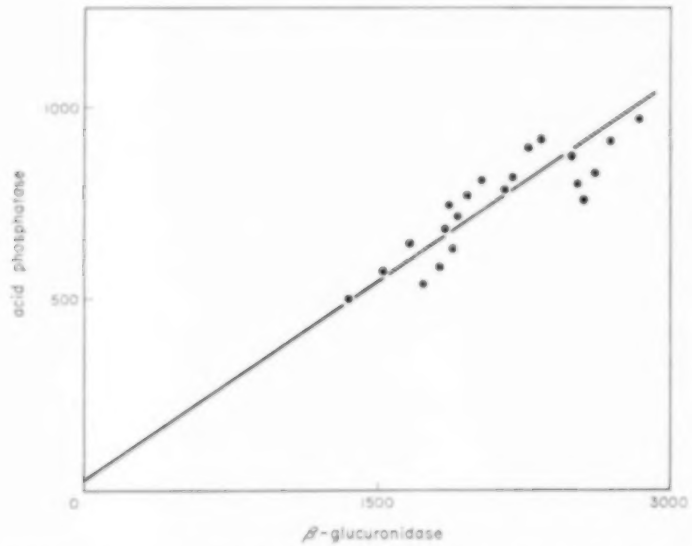

FIG. 2. Correlation between acid phosphatase activity and β-glucuronidase activity per 100 mg wet spleen tissue of the first three groups of mice. Acid phosphatase activity: μg P/100 mg wet spleen per hr. β-Glucuronidase activity: μg phenolphthalein/100 mg wet spleen per hr.

This correlation could not be statistically demonstrated for either the liver or the spleen in the four groups of mice which were injected with macromolecular substances.

Finally, it may be remarked that the rise in acid phosphatase activity observed in the present investigation is of the same order of magnitude as the rise in acid phosphatase activity described in a previous investigation in which Swiss mice were used.[4]

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THE REACTION BETWEEN PYRIDINE-4-ALDOXIME AND METHIODIDE THIOESTERS: A MODEL FOR ENZYMIC TRANSACETYLATION*

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Abstract—Oximes are useful therapeutic adjuncts to atropine in the treatment of anticholinesterase poisoning, although their exact mechanism is not fully understood. Since it can be shown that oximes react rapidly and reversibly with thioesters *in vitro*, it is suggested that part of their therapeutic effect may be due to an interference with acetylcholine synthesis (a thioester-dependent reaction). Rate constants for the reactions of pyridine-4-aldoxime methiodide (4-PAM) with a series of thioesters (forward reactions) and of the acetate of pyridine-4-aldoxime methiodide (Ac-4-PAM) with the corresponding mercaptans (reverse reactions) are reported. The forward reactions are first-order with respect to the thioester and oximate ion concentration; the reverse reactions are first-order with respect to the aldoxime ester and mercaptide ion concentrations. Equilibrium constants calculated from kinetic data agree with those estimated from an analysis of reactants and products at steady state. Equations are given, relating the reactivity of a thioester with the acid dissociation constant of its corresponding mercaptan, which are useful for predicting the rate of a reaction of thioester with pyridine-4-aldoxime methiodide and in selecting a thioester for maximum reactivity at a given pH. At physiological pH (7·4), a thioester whose corresponding mercaptan has a pK_a of about 8·2 will have maximum reactivity with 4-pyridine aldoxime methiodide.

THE effectiveness of pyridine-2-aldoxime methiodide (2-PAM) and certain other oximes as adjuncts to atropine in the therapy of organophosphate poisoning[1] has aroused interest in the mechanism of action of these oximes. While it has been shown that these materials *in vitro* are reactivators of organophosphate-inhibited cholinesterase, and that part of their effectiveness *in vivo* can be ascribed to such action,[2, 3] still their beneficial effects in the treatment of intoxication by quaternary ammonium anticholinesterases[4] point to a more general mechanism of action. Furthermore, the observation that in treatment of organophosphate-poisoned patients with 2-PAM, restoration of neuromuscular activity is more rapid than regeneration of muscle cholinesterase, requires a mechanism not fully satisfied by cholinesterase reactivation.[4–6]

Cholinesterase regulates the level of acetylcholine, a chemical held vital for neuromuscular transmission,[7] which is formed by the choline acetylase (ChAc)-catalysed transacetylation reaction involving acetylated coenzyme A(CoA-S-Ac), a thioester, and choline.[8] In the presence of inactivated acetylcholinesterase, any action which might limit the production (or release) of acetylcholine would mimic the effect of a

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reactivator of inactivated enzyme. It had previously been observed that oximes (which do not react with acetylcholine) will react quite rapidly at physiological pH and temperature with the thioester, acetylthiocholine.[9] This suggested the possibility that oximes may act as competitive acceptors in transacetylation, and thus may interfere with the production of acetylcholine.

The present paper describes kinetic and equilibrium studies of the reaction between pyridine-4-aldoxime methiodide* (4-PAM) and various thioacetates and between the acetate of pyridine-4-aldoxime methiodide (Ac-4-PAM) and several mercaptans in dilute aqueous solution at 37 °C. The results of our studies indicate that a rapid reaction between oximes and thioesters may be expected to take place under physiological conditions.

EXPERIMENTAL

Oximes

Pyridine-4-aldoxime methiodide (m.p. 181 °C) and O-acetyl-pyridine-4-aldoxime methiodide (m.p. 158 °C) were prepared from pyridine-4-aldoxime (Aldrich Chemical Co) according to the method of Ginsburg and Wilson[10].

Thiols and thioesters

2-N-Diethylaminoethanethiol acetate hydrochloride was prepared from 2-N-diethylaminoethanethiol hydrochloride (96 per cent purity, Evans Chemetics, Inc.) by reaction at room temperature with redistilled acetic anhydride (10 per cent excess). After removal of unreacted anhydride and acetic acid, the ester hydrochloride was recrystallized from a benzene and ethanol mixture (m.p. 98–99·5 °C).

Anal. Calc. for $C_8H_{17}ONS.HCl$: C, 45·5; H, 8·0; N, 6·6. Found: C, 46·2; H, 8·0; N, 6·5.

S-Acetylthiophenol was prepared from redistilled thiophenol (b.p. 62·3°/15 mm) (Eastman Kodak Co) and freshly distilled acetyl chloride. After 16 hr at room temperature, the mixture was poured over cracked ice and extracted from sodium bicarbonate solution with diethyl ether and dried over sodium sulfate (anhydrous). After removal of ether at reduced pressure, the ester was distilled *in vacuo* (b.p. 78 °C;3 mm).

Anal. Calc. for C_8H_8OS: C, 63·2; H, 5·3. Found: C, 63·3; H, 5·4.

2-N-Di*iso*propylaminoethanethiol ($n_D^{32} = 1{\cdot}4654$), 2-N-di*iso*propylaminoethanethioacetate ($n_D^{23} = 1{\cdot}4732$) and allyl mercaptan were obtained from Dr. F. Hoffman, Chemical Research Division, Army Chemical Center, Md.

p-Methylthiophenol (m.p. 41·3 °C), *p*-chlorothiophenol (m.p. 51–3 °C) and thioglycolic acid (80 per cent in aqueous solution, analytical grade) were purchased from Eastman Kodak Co. Glutathione, S-acetylglutathione and l-cysteine hydrochloride were obtained from Nutritional Biochemicals Corp, and cysteine methyl ester hydrochloride (m.p. 145 °C) was prepared by Dr. R. E. Plapinger.[11] Acetylthiocholine bromide was purchased from LaWall-Harrisson Laboratories, Philadelphia, Pa.

Analytical methods

Iodometric titration. The mercaptan or thioester was weighed into a 100 ml volumetric flask containing KNO_3 (500 mg) and sequestrene-2 Na (Alrose Chemical Co)

* This oxime, although not as effective in therapy as the corresponding 2-substituted aldoxime, was chosen for these studies because of the greater stability of its derivatives. It is believed that the observations reported herein are, however, qualitatively applicable to 2-PAM and the trimethylene bis-4-pyridine aldoxime, TMB-4.

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(200 mg), and brought to volume with 0·05 M acetic acid–sodium acetate buffer of the desired pH. Two 10 ml aliquots were withdrawn, acidified with 0·5 ml of 0·1 N HCl, and the solutions were titrated with standard iodine solution (5×10^{-3} N), using a 1 per cent starch (soluble) solution as indicator (19). In the presence of sequestrene-2 Na, oxygen and trace metals do not interfere with the analysis. In a separate flask, acetyl-4-PAM (612 mg) or 4-PAM (528 mg) and KNO_3 (500 mg) were dissolved in 50 ml of 0·05 M acetic acid–sodium acetate buffer of the same pH and placed in a water bath at 37·0 ± 0·1 °C. (The concentration of acetyl-4-PAM (or 4-PAM) was 2×10^{-3} M).

To 50 ml of the mercaptan or thioester solution was added, with mixing, 50 ml of the oxime solution. Aliquots (10 ml) were removed periodically, acidified with 0·5 ml of 0·1 N HCl and immediately titrated with the standard iodine solution.

Spectral studies. Changes in mercaptan concentration determined iodometrically were correlated with the formation or disappearance of Ac-4-PAM measured spectrophotometrically. Reaction mixtures containing equal concentrations ($2{\cdot}5 \times 10^{-3}$ M) of mercaptan (DET) and acetylated oxime (Ac-4-PAM) or of thioester (DETA) and oxime (4-PAM), were incubated at 37 °C in acetate buffer (0·05 M, pH = 5·0). At frequent intervals, 2 ml aliquots were removed and acidified with hydrochloric acid (0·1 N). One portion was assayed for mercaptan iodometrically and a second portion was diluted fifty-fold for spectral measurement. At this concentration of oxime (5×10^{-5} M), the solution had an absorption of less than 1·0 optical density units at the desired wavelength (250 mμ). The amount (X) of Ac-4-PAM (moles/l.) present in the mixture at any time was then calculated from the following relationship:

$$\text{Observed optical density at } 250\ m\mu = \text{O.D. (Ac-4-PAM)} + \text{O.D. (4-PAM)}$$

$$\text{where } e_{4\text{-PAM}} = 0{\cdot}53 \text{ O.D. units at } 5 \times 10^{-5} \text{ M}$$

$$e_{\text{Ac-4-PAM}} = 0{\cdot}81 \text{ O.D. units at } 5 \times 10^{-5} \text{ M}$$

$$\text{and O.O.D.} = \frac{0{\cdot}81X}{5 \times 10^{-5}\ \text{M}} + \frac{0{\cdot}53\ (5 \times 10^{-5}\ \text{M} - X).}{5 \times 10^{-5}\ \text{M}}$$

The initial changes in Ac-4-PAM concentration are compared with mercaptan values in Table 1.

TABLE 1. RSH + Ac-4-PAM $\underset{\text{REVERSE}}{\overset{\text{FORWARD}}{\rightleftharpoons}}$ RSAc + 4-PAM

Time (min)	Forward direction		Reverse direction	
	RSH (moles/l. × 10^5)	Ac-4-PAM (moles/l. × 10^3)	RSH (moles/l. × 10^5)	Ac-4-PAM (moles/l. × 10^3)
0	2·50	—	0·05	0·00
18	1·77	1·77	—	—
28	1·63	1·52	—	—
30	—	—	0·13	0·10
57	1·30	1·32	—	—
62	—	—	0·25	0·21
80	1·20	1·16	—	—
90	—	—	0·36	0·34
150	—	—	0·54	0·70
163	1·14	1·16	—	—

TABLE 2. RATE CONSTANTS FOR REACTION OF 4-PAM AND DETA AT 37 °C IN 0·05 M ACETATE BUFFER AT DIFFERENT pH LEVELS

No. of runs	pH	4-PAM (Mx10²)	DETA (Mx10²)	k_{obs} (corr.)*† × 10³ (min⁻¹)	k'_{2f} (l. mole⁻¹ min⁻¹)	$k_{2f} \times 10^{-2}$ (l. mole⁻¹ min⁻¹)
2	5·0	2·0	0·625	6·50	0·325	10·3
4		1·0	1·25	3·16	0·316	10·0
2		2·0	1·25	7·20	0·360	11·4
2	5·5	2·0	0·625	23·66	1·18	11·8
2		2·0	1·25	21·4	1·07	10·7
2	6·0	2·0	0·625	80·66	4·03	12·8
1		2·0	1·25	62·26	3·11	9·9
						11·0 ± 0·7

* pH maintained constant by automatic addition of alkali.
† Corrections of first order rate constants (× 10³) for the spontaneous hydrolysis of DETA at pH 5·0, 5·5, 6·0 in 0·05 M acetate buffer are: 0·55, 0·54, and 1·14 min⁻¹.

TABLE 3. RATE-CONSTANTS FOR THE REACTION BETWEEN 4-PAMAc AND DET AT 37 °C IN 0·05 M ACETATE BUFFER AT DIFFERENT pH LEVELS

No. of runs	pH	4-PAMAc × 10²	DET × 10²	$k_{obs(corr.)}$* × 10² (min⁻¹)	k'_{2f}	$k_{2f} \times 10^{-3}$
1	5·0	1·0	0·625	3·82	3·82	2·71
2		1·0	1·25	3·50	3·50	2·48
2		2·0	1·25	8·82	4·41	3·13
2	6·0	1·0	0·625	36·5	36·5	2·61
						2·74 ± 0·20

* First-order rate-constants for the spontaneous hydrolysis of DETA at pH 5·0 and 6·0 in 0·05 M acetate buffer are: 0·55 and 1·14 × 10⁻² min⁻¹, respectively.

TABLE 4. RATE-CONSTANTS FOR THE REACTION BETWEEN 4-PAM (2 × 10⁻² M) AND ACETYLGLUTATHIONE (Ac-GSH)* AT 37 °C IN 0·05 M ACETATE BUFFER

No. of runs	pH	Ac-GSH × 10²	k_{obs}* × 10³ (min⁻¹)	k'_{2f} (l. mole⁻¹ min⁻¹)	$k_{2f} \times 10^{-2}$ (l. mole⁻¹ min⁻¹)
2	5·0	0·625	3·94	0·197	
1		1·250	3·77	0·189	6·12
1	5·96	0·625	35·7	1·79	6·23

* The spontaneous hydrolysis of Ac-GSH under these conditions was negligible.

TABLE 5. RATE-CONSTANTS FOR REACTION OF Ac-4-PAM (2 × 10⁻² M) AND GLUTATHIONE (GSH) AT 37 °C IN 0·05 M ACETATE BUFFER

No. of runs	pH	(GSH) M × 10²	k_{obs} × 10² (min⁻¹)	k'_{2f} (l. mole⁻¹ min⁻¹)	$k_{2f} \times 10^{-4}$ (l. mole⁻¹ min⁻¹)
3	5·0	0·625	2·89	1·45	2·30
1		1·250	3·15	1·58	2·50
1	5·45	0·625	7·10	3·55	2·00
2	5·85	0·625	18·75	9·38	2·10
1	5·95	0·625	22·0	11·0	1·95
					2·17 ± 0·18

RESULTS AND DISCUSSION

In solutions containing approximately equimolar concentrations of 4-PAM and 2-diethylaminoethanethioacetate (DETA), or of 2-diethylaminoethanethiol (DET) and the acetate of 4-PAM (Ac-4-PAM) the reaction approached a steady state (Table 1). Kinetic data obtained at constant pH (in acetate buffer), over a pH range of from 5·0 to 6·5, are shown in Tables 2 and 3. Analysis of rate data of pH 5 by a differential method[12] indicates that the reaction is bimolecular, the rate of reaction

TABLE 6. RATES OF REACTION BETWEEN 4-PAMAc (2×10^{-2} M) AND VARIOUS MERCAPTANS AT pH 5·0 AND 37 °C IN 0·05 M ACETATE BUFFER

Compound	Mercaptan $\times 10^3$	$k_{obs} \times 10^3$ (min^{-1})	k'_{2r} (l. mole^{-1} min^{-1})	$k_{2r} \times 10^{-3}$ (l. mole^{-1} min^{-1})	pK_a of mercaptan (25 °C)
p-Chlorothiophenol	1·25	6·72*	3·36		
	0·625	6·83*	3·42	0·0329	5·94†
Thiophenol	1·25	7·5*	3·75	0·252	6·82†
p-Methylthiophenol	1·25	10·25*	5·13	0·377	6·86†
Cysteine methylester	1·25	4·34	2·17		
	0·625	4·20	2·10	0·602	7·45‡
2-Di*iso*propylamino-ethanethiol	0·625	10·4	5·20		
	1·25	10·3	5·15	2·60	7·70†
2-Diethylaminoethanethiol	—	—	—	2·74§	7·85
Glutathione	—	—	—	21·7§	9·20**
Thioglycolic acid	1·25	1·70	0·85	177·5	10·32**
	0·625	1·70	0·85		
Allylmercaptan	1·25	6·42	3·21	ca. 3×10^4	ca. 12††

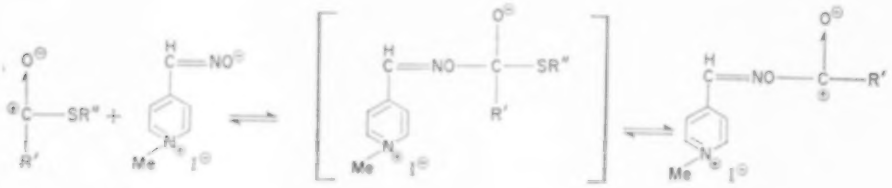
* Kinetic study in aqueous ethanol (20 per cent ethanol by volume) solution.
† Determined by potentiometric titration; thiophenols titrated in aqueous-acetone (20 per cent acetone by volume) solution.
‡ Based on value for cysteine ethylester given in footnote **.
§ Average value in 0·05 M Ac^- buffer, taken fom preceding tables.
** R. Benesch and R. Benesch, *J. Amer. Chem. Soc.* **77**, 5877 (1955).
†† Estimated from data given by H. C. Brown, D. H. McDaniel and O. Haflinger, *Determination of Organic Structures by Physical Methods*, Academic Press, New York (1955).

TABLE 7. RATES OF REACTION BETWEEN 4-PAM (2×10^{-2} M) AND VARIOUS THIOESTERS IN 0·05 M ACETATE BUFFER AT pH 5·0 AND 37 °C

Compound	Thioester conc. (moles/l. $\times 10^3$)	$k_{obs} \times 10^3$ (min^{-1})	k'_{2r} (l. mole^{-1} min^{-1})
DETA			0·33*
Acetylglutathione	—	—	0·19†‡
Acetylthiocholine	0·625	8·5	0·425‡
Acetylthiophenol	0·55	10·0	0·50‡
DITA§	1·25	5·0	0·255‡

* Average value taken from data at pH 5·0, 0·05 M acetate buffer, 37 °C, given in Table 2.
† Average value taken from data at pH 5·0, 0·05 M acetate buffer, 37 °C, given in Table 4.
‡ No correction is made for the spontaneous hydrolysis of the esters. Under the conditions of the experiments, the spontaneous hydrolysis was negligibly small.
§ DITA = 2-Di*iso*propylaminoethane thioacetate.

being proportional to the first powers of the reactants. The constancy of the true bimolecular rate-constants (k_{2f} and k_{2r}), in contrast to the variability of pseudo-bimolecular rate-constants (k'_{2f} and k'_{2r}), the direct dependence of the rate in either direction upon the concentrations of reactants raised to the first powers, and the steady state analysis, suggest that the reaction may be depicted as:



A similar dependency of rate upon pH was found in studies of the reaction between glutathione and its ester with Ac-4-PAM and 4-PAM (Tables 4 and 5). Kinetic data of the reaction between 4-PAMAc and nine mercaptans, at pH 5·0 in 0·05 M acetate buffer, are shown in Table 6. Data on the reaction between 4-PAM and five thioesters are shown in Table 7.

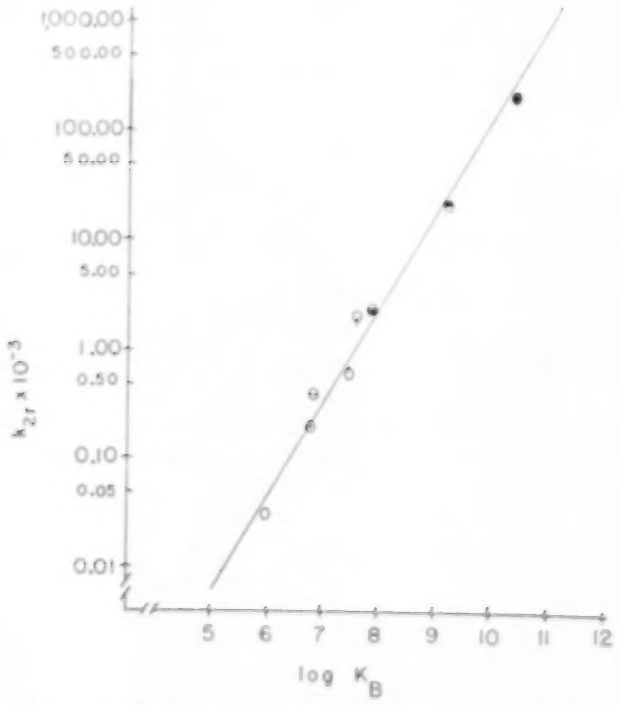


FIG. 1. Bronsted relationship between the true bimolecular rate constant (k_{2r}) and the basicity of several mercaptans reacting at pH 5·0 with acetyl-4-PAM: *p*-Chlorothiophenol, ○; thiophenol, ⊙; and *p*-methylthiophenol, ⊖; cysteine methylester, ◇; 2-N-di*iso*propylaminoethanethiol, ♀; 2-N-diethylaminoethanethiol, ◒; glutathione, ◓; and thioglycolic acid, ●.

Although the basicities of the mercaptide ions (Table 6) vary by as much as four powers of ten, the observed first-order constants (in the pH region in which a linear relationship exists between the ion concentration and pH) are all of the same order of magnitude. This implies that a Bronsted type of relationship exists between the true bimolecular rate constants and the basicities of the nucleophile (Fig. 1). The equation relating the bimolecular rate constant and the basicity of the anion, calculated by the method of least squares, is:

$$k_{2r} = 3{\cdot}39 \times 10^{-4} (1/K_A)^{0{\cdot}864}$$

where K_A is the acid dissociation constant. For the true bimolecular rate constant (k_{2r}), in the equation showing the relationship between this constant and the basicity of the mercaptide ion, is substituted its equivalent $k'_{2r}(H^+/K_A + 1)$:

$$k'_{2r} = \frac{3{\cdot}39 \times 10^{-4} (1/K_A)^{0{\cdot}864}}{(H^+/K_A + 1)}$$

By differentiating k'_{2r} with respect to $1/K_A$, and setting the differential equal to zero, one obtains the relationship:

$$1/K_A = \frac{6{\cdot}35}{[H^+]}$$

from which at any pH, a value of K_A of the mercaptan of maximum reactivity may be calculated. At pH 7·4, and $K_A = 6{\cdot}25 \times 10^{-9}$, the bimolecular rate constant for a mercaptan with such a K_A can be calculated from the relationship:

$$k_{2r} = 3{\cdot}39 \times 10^{-4} (1/K_A)^{0{\cdot}864}$$

and, using the equation:

$$K_{eq} = \frac{[k_{2f} K_A/(H^+ + K'_A)]}{k_{2r} [K'_A/(H^+ + K'_A)]}$$

where K_A and K_A' are the acid dissociation constants of the 4-PAM and mercaptan, respectively, one can calculate k_{2f}. Equilibrium constants of several systems calculated from the rate data and determined experimentally are shown in Table 8.

For the thioester derived from a mercaptan of $K_A = 6{\cdot}25 \times 10^{-9}$, it can be estimated that the observed bimolecular rate constant (k'_{2f}) of the reaction between this

TABLE 8. EQUILIBRIUM CONSTANTS FOR REACTION:
RSAc + 4-PAM ⇌ 4-PAMAc + RSH

RSAc	k'_{2f} (l. mole^{-1} min^{-1})	k'_{2r} (l. mole^{-1} min^{-1})	$K_{eq} = k'_{2f}/k'_{2r}$	K_{eq} exp $= \frac{\text{(products)}}{\text{(reactants)}}$
DETA	0·33	3·91	0·085	0·128†
Acetylglutathione	0·19	1·51	0·126	0·136‡
Acetylthiophenol	0·50	3·75	0·133	0·126‡
DITA	0·255	6·55	0·039	0·038§

† Average of two determinations.
‡ Average of three determinations.
§ Average of three determinations.

thioester and 4-PAM will be of the order of 50 l. mole^{-1} min^{-1}. Thus, with concentrations of the oxime as low as 10^{-3} M, the thioester might be expected to be deacylated at a rate of 5 per cent per min. Although this paper describes an *in vitro* study and no *in vivo* experiments have been performed to show that the antidotal properties of oximes are actually due, in part, to an interference with acetylcholine synthesis, these calculations would indicate that such an occurrence is indeed plausible.

That oximes may exert a significant therapeutic action by interference with acetylcholine production (synthesis or release) rests with the acceptance of two premises: (a) oximes reach the site of acetylcholine synthesis; and (b) the oxime is of sufficient reactivity to compete with acetyl acceptors normally involved. Premise (a) appears reasonable if one assumes that cholinesterase and the enzymes associated with acetylcholine formation are close to one another in space, since *in vivo* the oximes have been shown to reactivate inhibited cholinesterase.

Premise (b) is more difficult to substantiate since the rate of acetylcholine formation *in vivo* is difficult to assess.* It can be stated, however, that reactions involving 4-PAM and a thioester, whose corresponding mercaptan has a pK_a of about 8·2, are relatively rapid. While the reaction rate at physiological pH will be a maximum for thioesters of such a mercaptan, nevertheless thioesters of mercaptans having values for pK in the range 7·5–9·2 will react at 80 per cent of this rate. The likelihood of mercaptans having such a pK range *in vivo* is tenable, in view of the known dissociation constants of mercaptans, e.g. pantetheine† (pK_a = 9·25), mercaptoethylamine (p$K_{1,2}$ = 8·35),[12] glutathione (pK_a = 9·2), 2-N-diethylaminoethanethiol (pK_a = 7·85).

The rate data given in Table 7 indicate that there is no marked effect of thioacetate structure upon its reactivity with 4-PAM. This is in agreement with observations by Tarbell *et al.*[14] on the rates of ammonolysis and of hydrolysis of various thioacetates and in contrast to the observations of Noda *et al.*,[15] who found appreciable effects of structure on reactivity of thioacetates in reactions with hydroxylamine.

The importance of the mercaptide ion (rather than the undissociated mercaptan) in biological reactions has been pointed out by Benesch and Benesch.[13] In biochemical reactions involving a series of mercaptans having varying dissociation constants, the treatment of the data by the method shown herein, in order to predict at a given pH level, the mercaptan of maximum reactivity should prove useful in helping to explain the observed biological variations of drug potency.

* That oximes interfere with sulfanilamide acetylation catalysed by pigeon liver enzyme, a reaction involving acetylated co-enzyme-A, has been demonstrated since this study was completed (T. Wagner-Jauregg and H. Saner, *Arznei-Forsch.* **9**, 579 (1959).

† While pantetheine is most closely related structurally to co-enzyme-A, the pK_a of co-enzyme-A may actually be lower since it contains an amino group. That a neighboring group can influence sulfhydryl dissociation has been pointed out in the case of cysteine (p$K_{1,2}$ = 8·53) and its ethyl ester (p$K_{1,2}$ = 7·45) by J. T. Edsall and J. Wyman, in *Biophysical Chemistry*, Academic Press, New York (1958), p. 502. Pantetheine was prepared from pantethine (Calif. Corp. for Biochem. Research) by reduction over zinc and hydrochloric acid. Spectral absorption (λ = 234 mμ) was measured at pH = 1·0, 7·89 and 9·29, and the pK_a was estimated graphically by the method of Marsh and Saville (personal communication).

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PREVENTION OF THE CONVULSIVANT AND LETHAL EFFECTS OF *ISO*NICOTINIC ACID HYDRAZIDE BY PYRUVIC ACID

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Abstract—The effect of *iso*nicotinic acid hydrazide upon the concentration of pyruvic acid in the serum of rabbits injected with high doses of the drug, and the prevention of the toxic effects of the hydrazide by means of pyruvic acid were studied.

It was found that *iso*nicotinic acid hydrazide reduces the amount of circulating pyruvic acid, combining with it to form an *iso*nicotinyl-hydrazone. The simultaneous injection of sodium pyruvate prevented both the convulsant and the lethal effects of the hydrazide. This was attributed to the reduction of its concentration to levels under the LD_{50}, through the formation of the hydrazone and to the maintenance of the circulating pyruvate at safe concentrations.

INTRODUCTION

In a recent paper Balzer *et al.*[1] have shown that *iso*nicotinic acid hydrazide (INH) reduces the levels of γ-aminobutyric acid (GABA) and glutamic acid (GA) in the brain of mice. The reduction in GABA had been previously related to the inhibition of GA-decarboxilase,[2] probably through the formation of the *iso*nicotinyl-hydrazone of pyridoxal (pyridoxal · INHzone).[3] The simultaneous injection of pyridoxine led to a still greater reduction in GABA levels,[1] as it was to be expected since it would enhance the formation of the INHzone, but GA concentrations reverted to normal values. The toxic effects of INH were not prevented by pyridoxine, and they were attributed to a reduction in the turnover of GA.

A partial protection against high doses of INH was obtained by della Pietra *et al.*[4] with the simultaneous administration of GA, complete protection being obtained with GA plus pyridoxine.

It is known[5] that INH reacts with pyruvic acid to form an hydrazone (pyruvic acid · INHzone), and *in vitro* experiments[6] demonstrated that INH causes a reduction in the amount of pyruvic acid in tissues. This indicates the possibility of INH inhibiting GA biosynthesis through the removal of pyruvic acid and would explain the fact of pyridoxine reverting GA levels to normal values.

The present work was undertaken in order to ascertain the effect of INH upon the levels of pyruvic acid in the blood of rabbits and the prevention of the convulsivant and lethal effects of INH by pyruvic acid.

MATERIALS AND METHODS

INH and pyruvic acid were injected intravenously into rabbits, and blood samples were collected from the marginal vein of the ear at different times after the injection.

"Total" pyruvic acid

We found that the technique of Friedeman and Haugen,[7] when used for the determination of pyruvic acid in the serum of INH-treated animals, assays also for the INH-bound pyruvate, so giving a "total" reading. This would explain the results quoted by Chmelev,[8] who found an accumulation of pyruvic acid in the blood of INH-treated patients.

"INH-bound" and "free" pyruvic acid

Pyruvic acid combined as an INHzone was evaluated by spectrophotometry (267 mμ), after being separated from the other metabolic derivatives by means of paper electrophoresis. Samples of 40 μl of serum were applied to a strip of filter paper (Macherey-Nagel no. 261), which was then impregnated with M/15 phosphate buffer

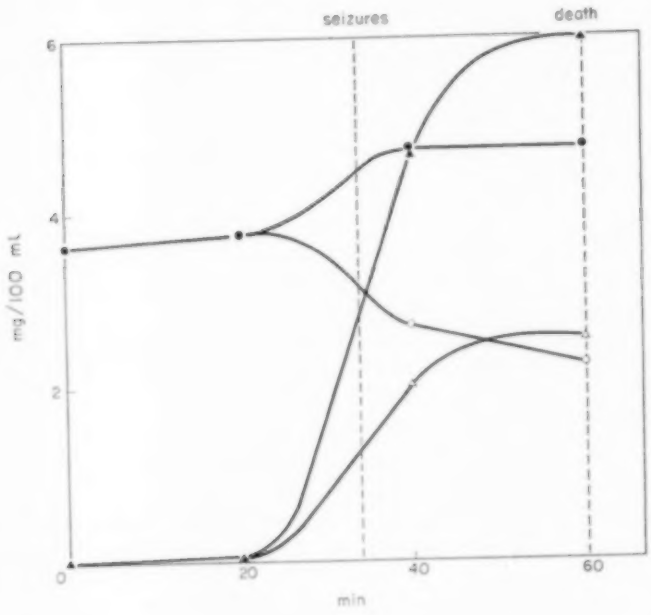

Fig. 1. Effect of an intravenous injection of INH (250 mg/kg body weight) upon the concentration of pyruvic acid in the serum of a rabbit. ●—● = "total" pyruvic acid; ○—○ = "free" pyruvic acid; ▲—▲ = Pyruvic acid . INHzone; △—△ = "INH-bound" pyruvic acid.

pH = 7·0 and submitted to a potential of 300 V during 3 hr. The INHzone of pyruvic acid migrates alone towards the anode, being easily localized by the observation of the dry paper strip under the u.v. lamp. The region of the paper containing the hydrazone was then cut and eluted overnight with water. The volume was adjusted to 3 ml and the absorption was measured at 267 mμ.

The amount of "INH-bound" pyruvic acid was calculated from the concentration found for the INHzone, and the "free" pyruvic acid was evaluated by subtracting the "INH-bound" from the "total" value.

RESULTS AND DISCUSSION

Fig. 1 shows the concentrations of "total", "free" and "INH-bound" pyruvic acid found in the blood of a rabbit at different times after the injection of 250 mg of INH per kg of body weight. After 1 hr there was a reduction of 39 per cent in the level of "free" pyruvic acid. Seizures started 35 min after the injection, when there was a 17 per cent reduction of circulating pyruvate. The last sample of blood was collected from the dying animal.

Assuming that seizures and death could have been caused by a shortage of available pyruvic acid, we injected it together with INH, when the following results were obtained (Table 1):

TABLE 1. PREVENTION OF THE CONVULSANT AND LETHAL EFFECTS OF INH (500 MG/KG BODY WEIGHT) IN RABBITS BY MEANS OF PYRUVIC ACID

(The theoretical amount of "free" INH was calculated assuming that all the Na pyruvate injected would combine to form the INHzone).

Rabbit no.	mg/kg body weight		Effect after . . . min		
	Na pyruvate	"Free" INH	Seizures	Death	Observations
1	—	500	50	90	—
2	—	500	50	70	—
3	—	500	30	60	—
4	80	400	80	120	—
5	80	400	30	50	—
6	160	300	60	100	—
7	240	200	40	60	—
8	240	200	50	90	—
9	320	100	—	—	None
10	320	100	—	—	None
11	320	100	—	—	None

Considering that the lethal dose (LD_{50}) of INH is 160 mg/kg body weight,[9] the prevention of the toxic effects of the drug by sodium pyruvate, as shown in Table 1, would depend on the reduction of the circulating "free" INH to levels under the LD_{50}, due to the formation of the *iso*nicotinyl-hydrazone.

The acute toxicity of the *iso*nicotinyl-hydrazone of pyruvic acid was studied in mice and in rabbits (unpublished results) and found to be nil even in doses of 10 g/kg of body weight (intraperitoneal injections). This reinforces the possibility of the anti-toxic activity of pyruvic acid in respect to INH being due to the formation of the INHzone, which would prevent the noxious reduction of blood pyruvate and consequent effect upon the metabolism of glutamic acid.

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MINIMUM CONCENTRATIONS OF N-METHYLPYRIDINIUM-2-ALDOXIME METHANE SULPHONATE (P2S) WHICH REVERSE NEUROMUSCULAR BLOCK

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Abstract—The effects of different concentrations of N-methylpyridinium-2-aldoxime methane sulphonate (P2S) on the neuromuscular block produced by methyl*iso*propoxy-phosphoryl fluoride (sarin), methyl*iso*propoxy-(2-dimethylaminoethylthio)-phosphine oxide (37 S-N) and methyl*iso*propoxyphosphoryl thiocholine (37 S-N$^+$) have been studied.

The block produced by the three inhibitors on the rat phrenic nerve diaphragm preparation is reversed by 1×10^{-5} M P2S. The time required to produce 50 per cent reversal of the block was the same against all inhibitors.

In anaesthetized cats poisoned with lethal doses of 37 S-N$^+$ plasma concentrations of about 2×10^{-5} M (4 μg/ml) were needed to counteract neuromuscular block, bradycardia, hypotension and respiratory failure.

ONE of the most effective antidote combinations against nerve gas poisoning is N-methylpyridinium-2-aldoxime with atropine. The oxime effectively ameliorates symptoms such as muscle weakness and paralysis thus decreasing the need for artificial respiration.[1] Plasma concentrations after intramuscular and oral administration of the oxime have previously been determined in man[2] but the concentrations needed for therapeutic effect, have not been established.

The aim of the present investigation was to determine the minimum concentrations of N-methylpyridinium-2-aldoxime methane-sulphonate (P2S) which reverse neuromuscular block caused by organophosphorous cholinesterase inhibitors.

MATERIALS*

P2S, N-methylpyridinium-2-aldoxime methane sulphonate was synthetized according to the method of Creasy and Green.[3] 37 S-N, methyl*iso*propoxy-(2-dimethylamino-ethylthio)-phosphine oxide acid oxalate and 37 S-N$^+$, methyl*iso*propoxyphosphory thiocholine iodide were synthetized according to the method of Tammelin,[4] sarin, methyl*iso*propoxyphosphoryl fluoride according to the method of Boquet,[5] and tabun, dimethylamidoethoxy-phosphoryl cyanide according to Holmstedt.[6]

METHODS

Rat phrenic nerve diaphragm preparation

The method was that described by Fredriksson and Tibbling.[7] If not otherwise stated the preparation was washed twice with Tyrode's solution before addition of

* The compounds were synthetized by Miss Inger Enander and Mr. Lars Fagerlind.

oxime in order to remove excess inhibitor. Control experiments showed that there was negligible reversal of twitch height after this washing procedure. The concentration of inhibitor in the bath was 5×10^{-7} M, which produced almost complete block within 30 min. As seen in Table 1 the time required for the development of maximum neuromuscular block, varies with the organophosphate. The preparation was consequently washed after 12 min in the case of sarin and after 30 min in the case of 37 S-N or 37 S-N$^+$ in order to obtain block of approximately the same magnitude. After washing P2S was added to a final concentration of from 5×10^{-6} to 1×10^{-3} M and the time required for the contractions to return to 50 per cent of their original value was then determined. It was found that the washing procedure did not significantly change the time required for 50 per cent reversal (in these experiments 5×10^{-4} M P2S was used).

TABLE 1. TIME REQUIRED FOR NEUROMUSCULAR BLOCKING EFFECT OF SARIN, 37 S-N, 37 S-N$^+$ AND TABUN 5×10^{-7} M ON THE ISOLATED RAT PHRENIC NERVE DIAPHRAGM PREPARATION

(The nerve was stimulated at 30 and 50 cycles/sec for 3 sec every 30 sec.)

Inhibitor	Time to maximum block in minutes (mean ± s.e.) Stimulation frequency	
	30 c/s	50 c/s
Sarin	6·7 ± 0·7 (n = 7)	7·3 ± 0·7 (n = 7)
37 S-N	21·6 ± 0·8 (n = 10)	22·8 ± 0·8 (n = 9)
37 S-N$^+$	16·5 ± 0·6 (n = 9)	17·1 ± 0·5 (n = 8)
Tabun	9·6 ± 0·7 (n = 5)	10·2 ± 0·8 (n = 5)

Frog rectus abdominis muscle

Receptor effects of the quaternary inhibitor 37 S-N$^+$ were tested according to Fredriksson and Tibbling.[8] The rectus muscle was incubated with sarin (5×10^{-5} M for 30 min) and then washed repeatedly. This concentration of sarin produces a complete inactivation of the cholinesterase of the muscle. 37 S-N$^+$ was then tested in increasing concentrations. Acetylcholine was used as reference.

Determination of therapeutic plasma concentrations

The experiments were performed on cats anaesthetized with sodium pentobarbital (30–40 mg/kg) intraperitoneally. Blood pressure, heart rate, respiration and contractions of the indirectly stimulated gastrocnemius–soleus muscles were recorded in all experiments. The recording technique was that described by Lindgren and Sundwall.[9]

A lethal dose of inhibitor was administered intravenously followed by intramuscular injections of P2S (in the biceps femoris of the leg opposite to that being stimulated) at different time intervals. Blood samples were taken from the femoral artery of the injected side before injection of organophosphate, at signs of improvement, after complete reversal of recorded symptoms and at the end of the experiment which usually lasted for from 2 to 3 hr. The samples (about 5 ml each) were collected in heparinized centrifuge tubes and the plasma analyzed for P2S.[2] Five millilitres of heparinized cat blood and 2 ml of Macrodex (R) were injected intra-arterially after each blood sample.

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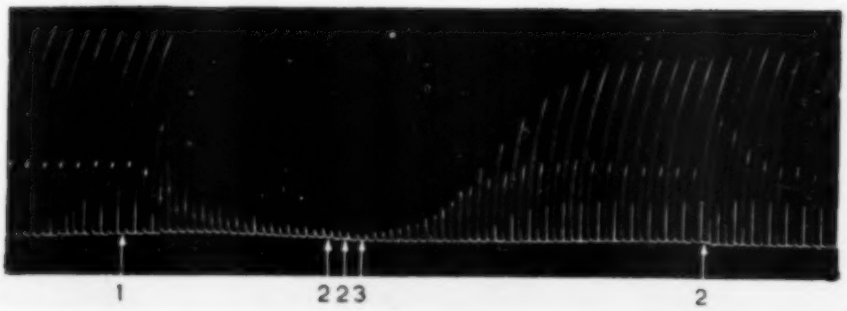

FIG. 2. Effect of P2S (5×10^{-5} M) on the neuromuscular block produced by sarin (5×10^{-7} M). Rat phrenic nerve diaphragm preparation. The nerve was stimulated with 30 and 50 c/s for 3 sec twice a minute. At (1) addition of sarin, at (2) washing and at (3) addition of the oxime.

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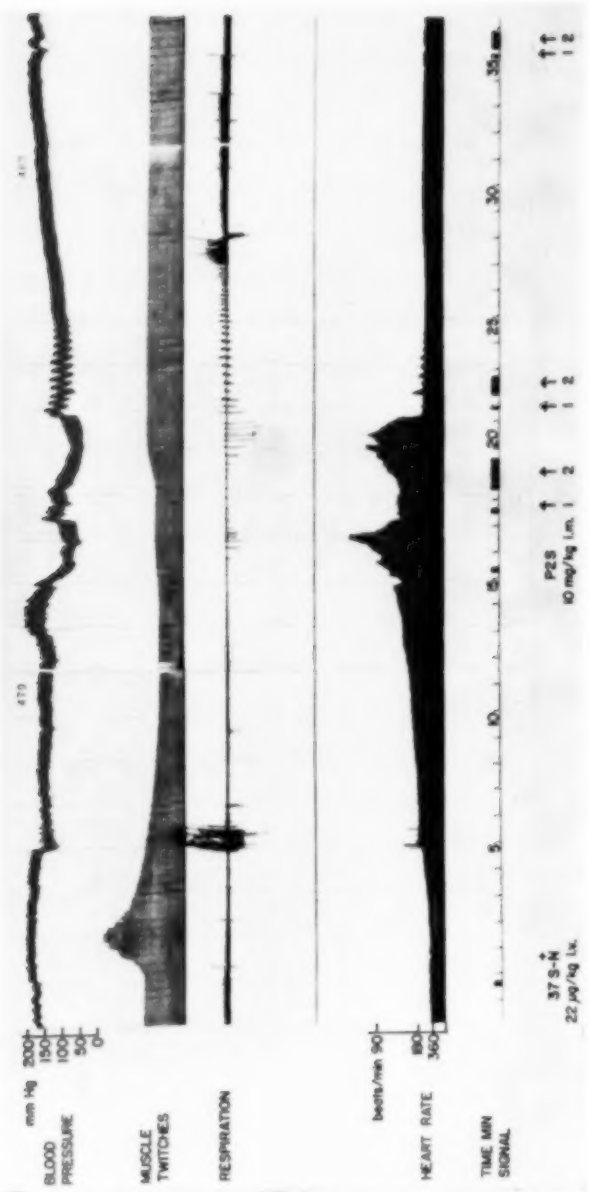

FIG. 3. Effect of P2S (10 mg/kg intramuscularly) on blood pressure, neuromuscular transmission, respiration and heart rate in anaesthetized cat poisoned with 37 S-N (22 μg/kg intravenously). At (1) collection of blood samples, at (2) injection of blood and macrodex. The concentrations of P2S in the samples were 8, 9 and 12 μg/ml plasma, respectively. The sciatic nerve was continuously stimulated with 0·3 c/s. Time in minutes.

RESULTS

Effect of P2S on neuromuscular block produced by sarin, 37 S-N, 37 S-N⁺ and tabun on rat phrenic nerve diaphragm preparation

The results are summarized in Fig. 1 and a typical experiment is shown in Fig. 2. It is seen that the 50 per cent reversal time was the same against sarin, 37 S-N and 37 S-N⁺ at oxime concentrations of 5×10^{-5} M to 5×10^{-4} M. At a concentration of 5×10^{-6} M, P2S failed to achieve 50 per cent reversal within 30 min in seven out of eighteen experiments and in the experiments where 50 per cent reversal was achieved, the time for this effect varied. It is also seen that the block produced by tabun is reversed much more slowly.

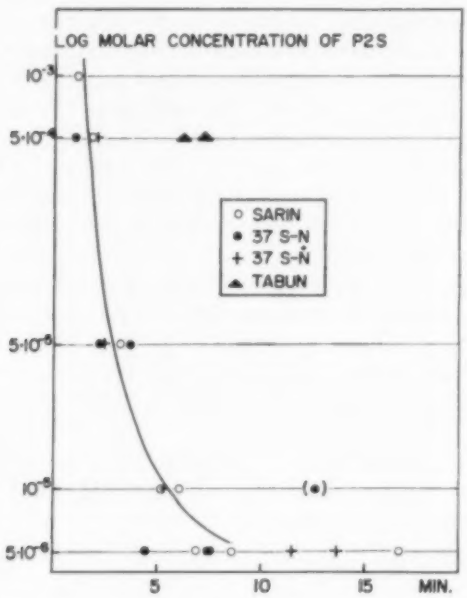

FIG. 1. Effect of P2S on time required for 50 per cent reversal of the neuromuscular block produced by sarin, 37 S-N, 37 S-N⁺ and tabun (5×10^{-7} M) (the curve is drawn for sarin). Rat phrenic nerve diaphragm preparation. The nerve was stimulated with 30 and 50 cycles/sec for 3 sec twice a minute.

Effects of 37 S-N⁺ on cholinergic receptors

The inhibitor evoked contractions of the sarin treated frog rectus abdominis muscle, but only at concentrations higher than 10^{-3} M. This concentration is much higher than might be expected in blood of animals poisoned by lethal doses. It is therefore less likely that receptor effects contribute to the toxicity of the drug.

Therapeutic plasma concentration of P2S in the anaesthetized cat

In preliminary experiments it was found that 22 μg/kg of 37 S-N⁺, given intravenously, is a lethal dose, death occurring within 18–25 min.

In three experiments 10 mg/kg of P2S was injected intramuscularly at the appearance of symptoms after intravenous injection of 22 μg/kg of 37 S-N⁺. Most toxic symptoms were reversed within 30 min and the plasma concentration of P2S was

from 4 to 12 μg/ml, when blood pressure, heart rate and respiration was normal. A typical experiment is shown in Fig. 3. P2S counteracts the severe bradycardia, re-establishes a normal blood pressure and spontaneous breathing. The increase in twitch height of the gastrocnemius muscle is simultaneous with the return of spontaneous respiratory activity. The plasma concentrations of P2S were about 8 μg/ml in the blood samples collected at the beginning of improvement and from 9 to 12 μg/ml when blood pressure, bradycardia and respiration became normal.

In two experiments the dose of 37 S-N$^+$ was increased to 27 μg/kg and P2S (10 mg/kg) was injected intramuscularly. The results were essentially the same as those obtained with the lower dose of inhibitor. The plasma concentration of P2S was about 8 μg/ml when blood pressure, heart rate and respiration became normal.

The dose of P2S was then decreased to 5 mg/kg in five experiments. In most cases the recorded symptoms were not fully reversed or were reversed only for a short time. The plasma concentrations were then from 2 to 4 μg/ml. A typical experiment is illustrated in Fig. 4. Four animals survived for more than 2 hr, one died after 30 min. No P2S was found in the plasma of the animal which died.

In the two first series of experiments above, neuromuscular block was generally not detected. In most cases only a potentiation of twitch height was seen before the injection of P2S. In the third series tetanic stimulation was performed instead of single twitches in order to obtain records on the effect of P2S on neuromuscular block. In these experiments the ability of the gastrocnemius to sustain a tetanus was decreased by from 20 to 95 per cent before injection of P2S. Improvement after P2S was always seen, but complete reversal only in one case.

In the experiments described bronchoconstriction and salivation were not measured, but it was noted that the dyspnoea and the increased flow of saliva were not relieved by P2S.

Experiments were also undertaken to demonstrate that the therapeutic effect of P2S could not entirely be due to an atropine-like effect (Fig. 5). Atropine prevents the bradycardia, but not the respiratory failure, in contrast to P2S which promptly restores normal respiration.

DISCUSSION

The results obtained indicate that plasma levels of P2S above 4 μg/ml counteract neuromuscular block *in vitro* and *in vivo*, and bradycardia, hypotension and respiratory failure *in vivo*. Atropine was not used in the experiments and it is therefore possible that the concentrations of P2S together with atropine needed for therapeutic effect might be lower, since atropine may prolong survival, thus giving the oxime more time to reactivate the inhibited enzyme.

If the results are valid in man, intramuscular injection of from 20 to 30 mg P2S per kg body weight should yield therapeutic plasma concentrations after from 5 to 10 min.[2] Effective plasma concentrations are also reached after oral administration, but as the rate of absorption is slow, the oxime must then probably be administered before poisoning.

P2S is equally effective against the neuromuscular block produced by sarin, 37 S-N and 37 S-N$^+$ (which yield the same type of phosphorylated enzyme) but is much less effective against the block produced by tabun. As it has been shown that methyl*iso*-propoxy phosphorylated cholinesterase is reactivated much more readily than that

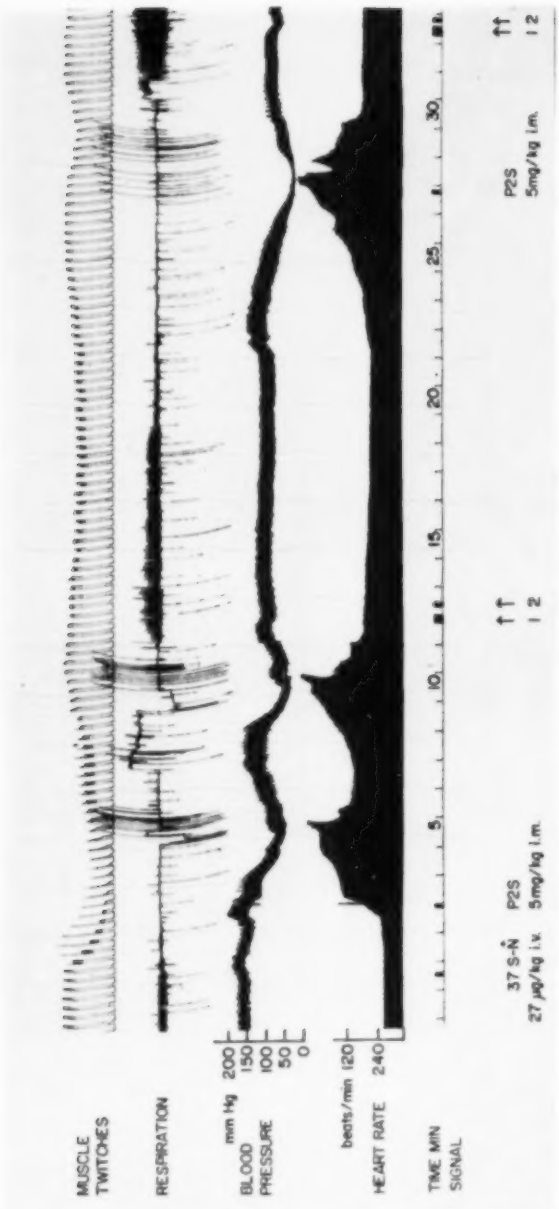

FIG. 4. Effect of P2S (5 mg/kg intramuscularly) on neuromuscular transmission, respiration, blood pressure and heart rate in anaesthetized cat poisoned with 37 S-N (27 μg/kg intravenously). At (1) collection of blood samples at (2) injection of blood and macrodex. Between the samples another intramuscular injection of 5 mg/kg of P2S was given. The concentrations of P2S was 2 and 3 μg/ml plasma, respectively. The sciatic nerve was stimulated with 20 c/s for 5 sec every 20 sec. Time in minutes.

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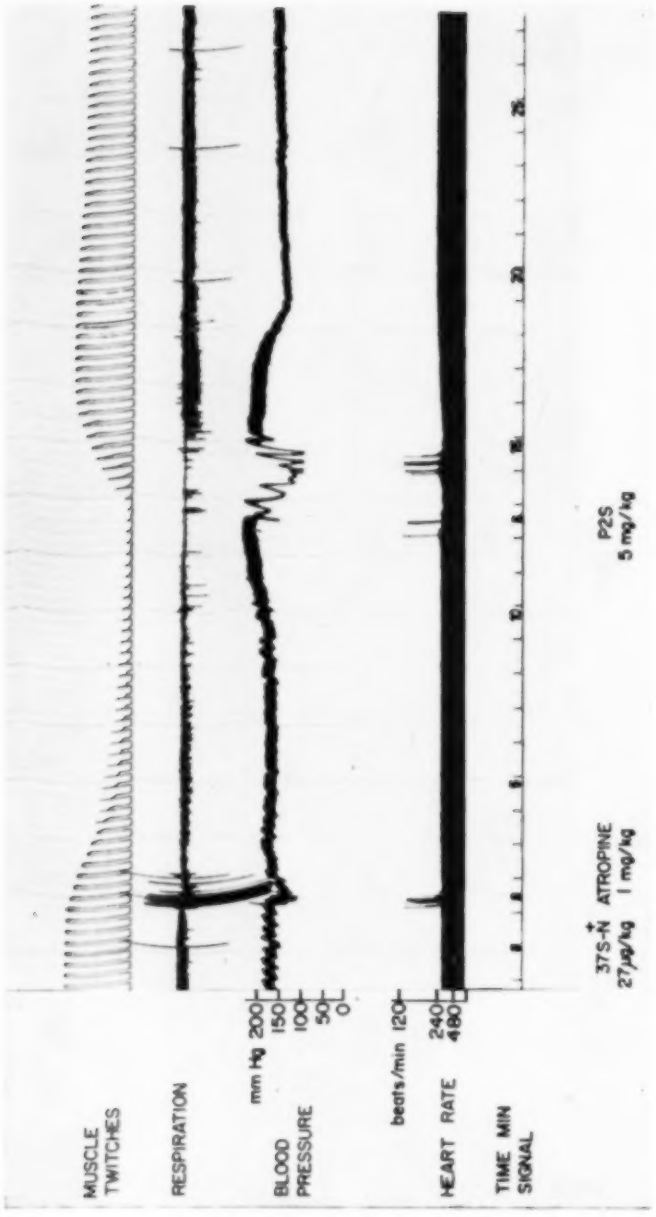

FIG. 5. Effect of P2S (5 mg/kg intravenously) on neuromuscular transmission, respiration, blood pressure and heart rate in anaesthetized cat poisoned with 37 S-N (27 μg/kg intravenously) and treated with atropine (1 mg/kg intravenously). The sciatic nerve was stimulated with 20 cycle/sec for 5 sec every 20 sec. Time in minutes.

inhibited by tabun[11] the experiments described support the concept that the oxime acts at the neuromuscular junction principally by reactivation of the phosphorylated cholinesterase.

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SHORT COMMUNICATIONS

On the mechanism of release of norepinephrine by α-methyl-*m*-tyrosine and α-methyl-*m*-tyramine

(*Received 25 September; accepted 7 October* 1961)

IT HAS recently been reported that α-methyl-3,4-dihydroxyphenylalanine (α-methyl-dopa) possesses hypotensive and sedative properties in man.[1] Biochemical studies in experimental animals have shown that α-methyl-dopa and the related compound, α-methyl-*m*-tyrosine, produce a marked and long-lasting lowering of norepinephrine levels in tissues (brain and heart)[2, 3] which could explain their pharmacological actions. However, the effects on norepinephrine do not arise as a result of inhibition of the biosynthesis of norepinephrine as might have been expected from the inhibitory actions of the two compounds on aromatic L-amino acid decarboxylase *in vitro*. The norepinephrine levels fall instead, through an effect on the ability of the tissues to take up or store the hormone.[2] Reserpine guanethidine and many sympathomimetic amines act in a similar manner.[4]

Studies with α-methyl-*m*-tyrosine and its decarboxylation product, α-methyl-*m*-tyramine, were undertaken in the guinea pig to determine the mechanism of the norepinephrine-release*; the amine is also a potent norepinephrine-releasing agent.[5]

TABLE 1. LACK OF CORRELATION BETWEEN NOREPINEPHRINE-RELEASE AND CONVERSION OF α-METHYL-*m*-TYROSINE TO ITS AMINE

	α-Methyl-*m*-tyramine in urine	Norepinephrine released (millimicromoles/g)	
	μg/24 hr	brain	heart
Normal	0	0	0
α-Methyl-*m*-tyrosine	470	1·3 ± 0·1	12·2 ± 0·7
α-Methyl-*m*-tyrosine following decarboxylase inhibitor	30	1·4 ± 0·1	10·8 ± 2·0
Decarboxylase inhibitor alone	0	0	0

α-Methyl-*m*-tyrosine (50 mg/kg) was administered intraperitoneally to guinea pigs and the animals were killed 24 hr later. The decarboxylase inhibitor, α-hydrazino-α-methyl-3,4-dihydroxyphenylpropionic acid (Merck, Sharpe and Dohme), was administered (25 mg/kg i.p.) 15 min before α-methyl-*m*-tyrosine and 30 min after. Each value for norepinephrine represents the average of findings obtained in from 3 to 5 individual animals.

It was found, first of all, that the norepinephrine-releasing action of α-methyl-*m*-tyrosine is not altered by agents which inhibit decarboxylation of the amino acid. In the experiment shown in Table 1, the decarboxylation of α-methyl-*m*-tyrosine was inhibited by the previous administration of α-hydrazino-α-methyl-3,4-dihydroxyphenylpropionic acid, which does not release norepinephrine from brain or heart. Although the formation of α-methyl-*m*-tyramine in test animals was diminished

* The term release is used for want of a better term. It is not meant to imply a mechanism for the lowering of tissue norepinephrine.

by more than 90 per cent, the amount of norepinephrine released was comparable to the amount released from control animals which received the α-methyl-*m*-tyrosine only. It would appear, therefore, that α-methyl-*m*-tyrosine is, by itself, an effective norepinephrine-releasing agent.

The availability of a sensitive and specific method for measuring α-methyl-*m*-tyramine in tissues* made it possible to investigate the stoichiometry of norepinephrine-release by the amine itself. The data in Fig. 1 show that, following its administration, α-methyl-*m*-tyramine reaches a level in heart about three times that of the control norepinephrine content. However, within a few hours, and lasting

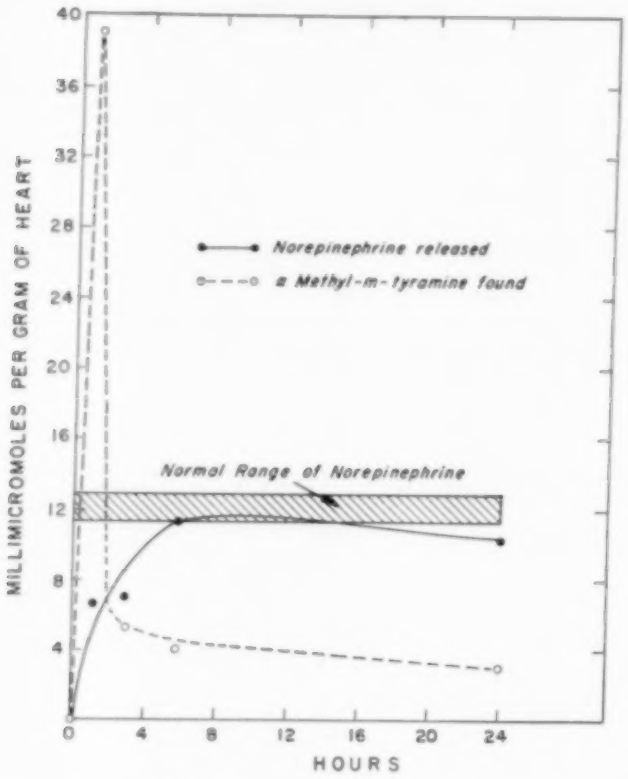

FIG. 1. Stoichiometry of the release of norepinephrine from guinea pig heart by α-methyl-*m*-tyramine. The drug (50 mg/kg) was administered intraperitoneally and individual animals were killed at the times indicated.

for at least 24 hr longer, when most of the norepinephrine had been released, the amount of α-methyl-*m*-tyramine in the brain had fallen to very low values, equivalent to only a fraction of the norepinephrine which was released. The levels of the tyramine-derivative at 5 and 24 hr may actually be lower than shown, since at these low levels the method is near the limits of its sensitivity. Similar data were obtained from studies of spleen and brain. It may be concluded that although the immediate release process may be a mole-for-mole displacement of norepinephrine by the administered α-methyl

* α-Methyl-*m*-tyramine was isolated by the same extraction procedure as is used for serotonin,[5] and was measured colorimetrically by the method described for *m*-tyramine by Mitoma *et al.*[6]

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m-tyramine, the longer lasting depletion cannot be explained on a displacement basis. A prolonged effect on the norepinephrine-binding sites or a slow rate of norepinephrine turnover in the tissues are more likely explanations. When α-methyl-*m*-tyrosine was administered in doses producing a maximal depletion of norepinephrine, barely detectable amounts of α-methyl-*m*-tyramine were found in the tissues.

Another conclusion from these studies is that α-methyl-*m*-tyramine is a more potent releaser of norepinephrine than is apparent when considerations are pased on the administered dose. The highest levels found in brain and heart, following dosages of α-methyl-*m*-tyramine which produced maximal norepinephrine-depletion (50 mg/kg), were about 1 and 8 μg/g, respectively. Based on the amounts of the drug actually reaching the tissues, α-methyl-*m*-tyramine and probably α-methyl-*m*-tyrosine and α-methyl-dopa may be as potent releasers of norepinephrine as are the Rauwolfia alkaloids. More detailed studies are now in progress.

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Increased intestinal absorption of foreign organic compounds in the presence of ethylenediaminetetraacetic acid (EDTA)

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FOREIGN organic compounds of low lipid-solubility are poorly absorbed from the intestine mainly because of their inability to penetrate readily the lipid-like membrane of the intestinal epithelial cells.[1] It is also recognized that most organic compounds used as drugs have too large a molecular size to pass freely through the aqueous "pores" of the epithelial cell membrane or through the spaces which might exist between the cells.[2] Recently, Windsor and Cronheim[3] have reported that two lipid-insoluble acids, heparin and sulfopolyglucin, which are normally very poorly absorbed from the gastrointestinal tract, are absorbed to an appreciable extent when administered together with the chelating agent, sodium EDTA. The present investigation of the effect of EDTA on the intestinal absorption of several lipid-insoluble compounds shows that the chelating agent increases the extent of absorption of neutral and basic compounds, as well as that of acidic compounds, and suggests a possible mechanism for this action.

Male Sprague-Dawley rats (200–250 g), fasted for 18 hr but allowed free access to water, were anesthetized with pentobarbital and ether, and the small intestine was exposed through an abdominal incision. Ligatures were placed at the pylorus and the ileo-cecal junction, and the bile duct was ligated. In most of the experiments, the renal pedicles were ligated so that the blood level of poorly absorbed drugs would be high enough to measure. Five ml of an isotonic salt solution, pH 7·4 (NaCl in 0·03 M phosphate buffer), containing either 1–5 mmoles of a drug per liter, or the drug together with 10 mg of disodium EDTA per ml, was introduced into the intestine through the pyloric ligature, and the abdominal incision closed. After 1 hr, the intestine and its contents were removed from the animal, blood was obtained by cardiac puncture, and the samples were assayed for the drug. The extent of absorption was calculated from the difference between the amount of drug placed in the intestine and that recovered from the intestinal tissue and contents after 1 hr. Sulfanilic acid was estimated by the colorimetric method of Bratton and Marshall,[4] and ^{14}C-labeled substances from the radioactivity of 1 N NaOH solutions of the samples. Each drug was studied in 8–12 animals.

The extent of absorption of two neutral molecules, mannitol-1:6-^{14}C and inulin-^{14}C, was markedly increased in the presence of EDTA. For example, when administered alone, these compounds were absorbed scarcely at all (less than 2 per cent), but, when administered with the chelating agent, the degree of absorption rose to values ranging from 7 to 11 per cent. The enhancement of absorption was also apparent from the plasma levels of radioactivity, which were increased 5- to 6-fold in animals that had received EDTA.

The absorption of a quaternary ammonium compound, decamethonium-N-methyl-^{14}C, was similarly increased in the presence of EDTA. Thus, control animals absorbed 2–3 per cent of the administered drug, whereas animals that had received the chelator absorbed 11–15 per cent. Moreover, EDTA-treated animals had plasma levels of the drug 5–6 times higher than those of control animals.

Two organic acids, sulfanilic acid and EDTA-2-^{14}C, also showed an increase in absorption in the presence of EDTA. For example, both acids were absorbed to the extent of 11–14 per cent in control animals (concentration of labeled plus non-labeled EDTA was 1 mg/ml), but the degree of absorption rose to values of 26–32 per cent in the presence of 10 mg of EDTA per ml, and the plasma levels of the drugs were increased about 5-fold.

It is conceivable that EDTA might alter the rate of absorption of certain acidic drugs by interfering with their chelation of calcium ion or other metal ions in the intestinal lumen. On the other hand, since EDTA increases the absorption of some neutral and basic compounds that would not be expected to combine with calcium, a less specific mode of action is indicated; for example, the chelating agent might alter the permeability of the intestinal epithelium. To investigate the latter possibility, we have measured the extent to which inulin passes from the bloodstream into the intestinal lumen when the lumen contains either saline solution alone, or the same solution with 10 mg of EDTA per ml. The amount of intravenously administered inulin appearing in the lumen within 1 hr was increased 4-fold in the EDTA-treated animals, indicating that the permeability of the blood-intestinal boundary had been increased. Perhaps the chelating agent acts by increasing the size of the membrane "pores" or by widening the spaces between the epithelial cells through the removal of calcium ions.

Further work is in progress to clarify the mechanism by which EDTA increases the absorption of lipid-insoluble compounds.

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Adaptation to ethanol in rats with special reference to brain tissue respiration

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THE respiration rate of brain cortex tissue stimulated electrically or with potassium chloride is reduced by ethanol[1] and by other depressants such as barbiturates.[2] Stimulated cortex slices are much more sensitive to the effect of these agents than are unstimulated ones.

It is well established that prolonged use of alcoholic beverages or other intoxicants will result in increased tolerance to these substances. Takemori[3] has demonstrated a tissue adaptation to morphine in rats, employing potassium-stimulation of brain cortex slices from animals given a course of morphine treatment. The present experiment was an attempt to discover whether adaptation to ethanol would be reflected in a reduction of the effect of this substance on electrically stimulated cortex tissue.

The experimental animals used were two groups of 20 six-month-old male rats (weight 300–400 g) from the laboratory stock. The rats were made to fast overnight. For measuring alcohol intoxication, the tilted plane technique[4] was used. First, the animals were tested when sober, and then each animal received 4 mg/g ethanol *per os* as a 25% (w/v) solution in tap water, and was tested at 20-min intervals for 100 min. The means of the lowest values observed for each individual are shown in Fig. 1 (A). The performance of the groups was similar. Then the experimental group was given 5 mg/g ethanol (without previous fasting) every other day for 20 days. The control animals received corresponding amounts of water. After the termination of this treatment, the effect of 4 mg/g ethanol after fasting overnight was tested again (Fig. 1 (B)). The difference between the groups was quite clear ($P < 0.001$, calculated by means of Student's *t*-test). Before the repeated treatment with ethanol the performance of the experimental group was reduced by 31·9 per cent, after it by only 16·7 per cent. The animals had acquired a tolerance to alcohol, whereas the performance of the control group was virtually unchanged.

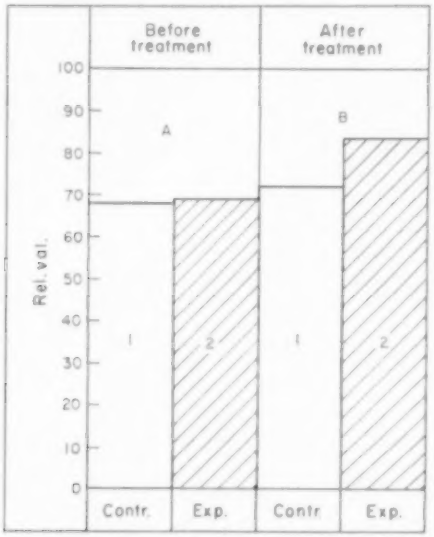

FIG. 1. The performance in the tilted plane test of two groups of 20 rats after administration of 4 mg/g ethanol. A, initial values; B, after repeated administration of water (control group) and 5 mg/g ethanol (experimental group). The performance of the same animals when not intoxicated is taken as 100 in both cases. The difference in the performances of groups 1 and 2 in B during intoxication was significant. ($P < 0.001$, s.d. ± 7·7 per cent in group 1; ± 8·5 per cent in group 2).

The administration of ethanol to the experimental animals was continued every second or third day in order to maintain their state of adaptation, until the animals were decapitated and slices prepared from the cerebral hemispheres for assay of the oxygen consumption. The method was as described earlier,[1] except that an electronic pulse generator was used for stimulation. The pulses were alternating condenser discharges, the frequency being 50 Hz, the peak voltage 12 v (10 mm distance between electrodes), and the time constant 0·6 msec. Stimulation was observed with an oscilloscope. As regards the magnitude and stability of the response, and the effect of ethanol, the results obtained with this apparatus are entirely comparable with those obtained with the earlier one.[1]

Three slices (60–80 mg) from one cerebral lobe were used as controls and the corresponding slices from the other lobe incubated in the presence of 0·130 M (0·6%) ethanol, the right and left lobes being used alternately as controls. Respiration during 60 min after an initial equilibration period of 18 min served as the measure of stimulation. Since controls and experimental slices were from the same brain, the *t*-test for correlated measures was used in the statistical evaluation of the results.

TABLE 1. DEPRESSING ACTION OF 130 MM (0·6%) ETHANOL ON THE RESPIRATION OF STIMULATED BRAIN TISSUE OF RATS AFTER REPEATED ADMINISTRATION OF WATER (CONTROL) AND 5 MG/G ETHANOL (EXPERIMENTAL)

Group	No. of animals	Stimulated oxygen uptake μmoles/g fresh weight/hr		Effect of ethanol $P <$	Percent inhibition
		No ethanol	130 mM ethanol		
Control	13	191·8 ± 12·5	169·3 ± 21·5	0·005	11·7
Experimental	18	189·2 ± 17·5	171·4 ± 18·7	0·01	9·4

No significant difference between the groups in respect of the action of ethanol was observed.

As shown in Table 1, the respiration of both the tissue from the control animals and that from the rats treated with ethanol was clearly depressed by ethanol. The slight numerical difference in the effect of ethanol on the controls (reduction 11·7 per cent) and on the experimental group (reduction 9·4 per cent) after this fairly short treatment was far from significant, however. That the acquired overall physiological tolerance to the effects of ethanol was not reflected in the experiments with brain slices may be due to the roughness of the method. It is thus felt that the present experiment still does not exclude the possibility of changes in cerebral cellular characteristics due to repeated treatment with ethanol.

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Note added in proof: After this communication had gone to press, the authors found that A. F. HOGANS, O. M. MORENO and D. A. BRODIE (*Amer. J. Physiol.* **204**, 434, (1961)). report behavioural tolerance to alcohol in monkeys after as little as 4 times of intravenous administration (2 g/kg). This tolerance was not parallelled by diminished effects on EEG-patterns recorded with chronic electrodes from the cortex, suggesting that subcortical centers may be involved in the early stages of nervous adaptation to ethyl alcohol.

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